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# EXERCISE AND FREE RADICAL INDUCED DAMAGE TO HUMAN SKELETAL MUSCLE

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*Dedicated to my mother Keleta.*

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Abstract

This thesis reports five novel studies investigating exercise and free radical induced damage to human skeletal muscle, with a review of related literature.

It has been proposed muscle adapts to eccentric exercise by increasing its resting length. The first study investigated the effects of manipulating knee extensor (KE) length during eccentric actions, on indices of muscle damage and knee angle specific force production. This study provided evidence that exercise performed at a long muscle length increased the damage susceptibility of the KE muscles, and produced a transient increase in muscle length.

Investigations using animals suggest the generation of high muscle forces contributes to initial injury. Surprisingly this effect has not been studied using a well controlled methodology in humans. The second investigation manipulated peak force during eccentric actions, using electrical myostimulation, and provided evidence that peak force was an important mechanical factor in initial muscle injury.

In rodents, exercise myopathy can compromise muscle antioxidant status, possibly resulting in free radical mediated injury. The third study investigated if these events occurred in human muscle, following eccentric exercise. Muscle myopathy did not compromise muscle antioxidant status or elevate lipid peroxidation indices 4 days and 7 days after exercise, suggesting free radicals did not contribute to muscle damage at these times.

In the previous study functional measures were not collected in the days following exercise and biochemical data were not collected in the 2 days following exercise. Therefore the fourth study evaluated indices of muscle damage and lipid peroxidation indices in blood, to determine if free radicals might contribute to delayed indices of muscle damage. Evidence of increased lipid peroxidation in the first 2 days after exercise, suggest free radicals might contribute to delayed muscle damage.

The effects of high intensity aerobic exercise on serum antioxidant capacity have not been reported previously. The final study showed running a half marathon increased serum antioxidant capacity, although this did not prevent lipid peroxidation or myocellular and lysosomal enzyme release.

# Contents

## List of figures

## List of tables

## List of abbreviations

| | |
|---|---|
| ANOVA | Repeated measures analysis of variance |
| βG | β-Glucuronidase activity |
| BLa | Blood lactate concentration |
| CAT | Catalase |
| CK | Serum creatine kinase activity |
| Cu | Copper |
| CV | Co-efficient of variation |
| Da | Dalton |
| DMSO | Dimethyl sulphoxide |
| ELISA | Enzyme linked immuno-assay |
| EM | Electron microscopy |
| Equ. | Equation |
| Fe | Iron |
| Fig. | Figure |
| G6PDH | Glucose-6-phosphate dehydrogenase |
| GPx | Glutathione peroxidase |
| GSH | Reduced glutathione |
| GSSG | Oxidised glutathione |
| HPLC | High pressure liquid chromatography |
| HRP | Horseradish peroxidase |
| IL | Interleukin |
| IU | International units |

| | |
|---|---|
| KE | Knee extensor |
| $L^{\bullet}$ | Lipid radical |
| LFF | Low frequency fatigue |
| LM | Light microscopy |
| $LO_2^{\bullet}$ | Peroxyl radical |
| MDA | Malondialdehyde |
| ML | Muscle length |
| Mn | Manganese |
| MS | Muscle soreness |
| MVC | Maximum voluntary contractile force |
| MVS | Maximum voluntary contractile force with PES |
| NS | Non significant |
| PES | Percutaneous electrical myostimulation |
| rad. | Radian |
| Rep. | Repetition |
| RF | *Rectus femoris* |
| ROS | Radical oxidative stress |
| SL | Sarcolemma |
| SOD | Superoxide dismutase |
| SR | Sarcoplasmic reticulum |
| TAC | Total antioxidant capacity |
| TBA | Thiobarbituric acid |
| Trolox Eq. | Trolox equivalents |

| | |
|---|---|
| UA | Serum uric acid concentration |
| Zn | Zinc |

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Finally and perhaps most importantly I must thank the subjects who participated in what have been termed "heroic studies in muscle damage", giving their time freely without financial or health benefits.

# CHAPTER 1

## Introduction

1.0.1 Exercise and free radical damage: Possible connections.

Physical exercise has many beneficial effects, although there is also evidence it increases the production of free radicals. A wealth of literature exists on the potentially damaging effects of such compounds (Ames 1989, Aust *et al.* 1993, Beal 1996) and excessive free radical production is characteristic of several disease states. The presence of free radicals cannot always be viewed as an undesirable event however, as they have many beneficial effects in relation to cellular signaling (Allen 1991) and immune function (Babior *et al.* 1984, Weiss 1989).

Humans possess a complex antioxidant defence system which involves the prevention of free radical formation, and the interception of radicals with antioxidants. Several reports suggest the balance between free radical formation and antioxidant defences could be perturbed during exercise (Singh 1992, Witt *et al.* 1992). Intense aerobic exercise and exercise myopathy are two conditions which may disturb this balance in muscle. In each case the overproduction of free radicals has the potential to produce oxidative injury. This thesis documents a series of experiments relating to exercise induced muscle damage and the possible involvement of free radicals in the damage process.

The terms injury and damage are commonly used in the exercise literature without an adequate definition or explanation. In recognition of the difficulty in defining injury Henson and Johnston (1987) stated : -

> "It is easy to fall back on the obvious requirement that injury must represent structural alteration of a given degree, e.g., lysis of cells, denudition of epithelium, blockage of a vessel or duct, or breakage or erosion of connective tissue elements. However, injury or damage in the clinical setting is much more subtle than this, and may often be manifested in functional changes without obvious structural counterparts. To the other extreme, functional damage can only be defined in the context of what is normal....."

For these reasons the terms damage and injury are used in reference to the pre-exercise condition, which is considered normal. Clearly this definition can present problems, for example changes in muscle contractility could result from muscle fatigue or muscle damage.

1.0.2 Free radical chemistry

The atomic orbitals of most biological molecules contain pairs of electrons; in contrast to free radicals, which contain one or more unpaired electrons (Halliwell & Chirico 1993). Nearly all chemical bonds are comprised of two electrons, which are usually broken asymmetrically by heterolysis. In this situation both electrons are located with a single fragment. When a bond is broken symmetrically (homolysis) a single electron stays with each fragment thereby forming two radicals (Equ. 1). Conversely, when two radicals combine their unpaired electrons, they form a covalent bond (Equ. 2). In the following equations a dash is used to represent a covalent bond and a dot ($^{\bullet}$) is used to designate the presence of one or more unpaired electrons.

Homolysis $\quad A\text{-}A \Rightarrow A^{\bullet} + A^{\bullet}$ (Equ. 1)

$A^{\bullet} + A^{\bullet} \Rightarrow A\text{-}A$ (Equ. 2)

As most biological molecules are not radicals, the probability of two radicals combining by chance, to form a stable compound is low. Thus when a radical gives one electron to, takes one electron from, or simply adds to a compound which is not a radical, that compound also becomes a radical. In biological systems, reactions between free radicals and non radicals usually proceed as chain reactions i.e. one radical forms another. Lipid peroxidation is the most studied and biologically relevant free radical chain reaction (Halliwell & Chirico 1993).

Highly reactive radicals such as the hydroxyl radical ($OH^{\bullet}$) frequently modify biological molecules by abstracting hydrogen. As the hydrogen atom has one proton and a single electron its removal from a molecule leaves an unpaired electron on the atom to which the hydrogen was originally attached. Thus, removal of a hydrogen atom can initiate lipid peroxidation (Halliwell & Chirico 1993).

$$L\text{-}H + OH^{\bullet} \Rightarrow H_2O + L^{\bullet} \quad \text{(Equ. 3)}$$

The mitochondrial electron transport chain results in more than 95% of the oxygen forming ATP and water. However it has been estimated 2 to 5% forms superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide ($H_2O_2$) as a

normal feature of aerobic metabolism (Pryor 1986). These reactive oxygen species are formed by what have been termed 'metabolic leaks' (Boveris & Chance 1973, Sjodin *et al.* 1990). Although $O_2^{\bullet -}$ and $H_2O_2$ are much less reactive than $OH^{\bullet}$ they still have the potential to produce cell damage. Transition metals (e.g. $Fe^{2+}$ and $Cu^{+}$) can react with $H_2O_2$ to form $OH^{\bullet}$ in Fenton and Haber Weiss reactions. In a simple form the Fenton reaction can be written : -

$$Fe^{2+} + H_2O_2 \Rightarrow OH^{\bullet} + OH^{-} + Fe^{3+} \qquad \text{(Equ. 4)}$$

This reaction can also be catalysed by copper.

$$Cu^{+} + H_2O_2 \Rightarrow OH^{\bullet} + OH^{-} + Cu^{2+} \qquad \text{(Equ. 5)}$$

Ferric complexes e.g. $Fe^{3+}$ do not react readily with $H_2O_2$ (Halliwell & Chirico 1993). The presence of reducing agents (e.g. ascorbate) are required to facilitate the conversion of Fe(III) to Fe(II) as shown in (Equ. 6), which can then participate in the Fenton reaction (Equ. 4).

$$Fe^{3+} + \text{ascorbate} \Rightarrow Fe^{2+} + \text{semidehydroascorbate}^{\bullet} \qquad \text{(Equ. 6)}$$

Therefore, environments containing free iron or copper, ascorbate and $H_2O_2$ have potential to produce damaging $OH^{\bullet}$ radicals.

### 1.0.3 Neutrophil derived reactive oxygen species

Exercise which produces substantial muscle damage can result in cellular infiltration in the affected tissues (Jones *et al.* 1986, Round *et al.* 1987). Neutrophils are typically involved in inflammatory processes, and may represent a major source of free radicals within damaged tissue. The biochemical pathways for the generation of reactive oxygen species is outlined below, but has been reviewed in detail by Weiss (1989).

The NADPH oxidase system is thought to generate at least three oxygen metabolites: $O_2^{\bullet -}$, $H_2O_2$ and $OH^{\bullet}$. This system is dormant in unstimulated neutrophils, but once triggered electrons are shuttled from

cytosolic NADPH to oxygen dissolved in the extracellular fluid. The typical reaction sequence is shown below.

$$2O_2 + NADPH \Rightarrow 2O_2^{\bullet -} + NADP^+ + H^+ \quad \text{(Equ. 7)}$$

In turn $H_2O_2$ can be generated from the dismutation of $O_2^{\bullet -}$, although very little $H_2O_2$ can be detected in the extracellular fluid (Test & Weiss 1984). As these oxygen species are relatively unreactive (Mao & Poznansky 1992), the ability of neutrophils to produce tissue injury using this pathway alone is limited. The possibility of forming $OH^{\bullet}$ with $Fe^{2+}$ (Equ. 4) also appears remote as neutrophils also release the iron binding protein lactoferrin.

Neutrophils appear to use the NADPH oxidase system in concert with myeloperoxidase (MPO) to produce powerful oxidants such as hypohalous acids. As $Cl^-$ is the concentrated halide *in vivo*, formation of hypochlorous acid (HOCl) is the most likely reaction, which is shown below.

$$H_2O_2 + Cl^- + H+ \xRightarrow{MPO} HOCl + H_2O \quad \text{(Equ. 8)}$$

The high concentration of MPO in neutrophils endows these cells with an enormous capacity for the production of hypochlorous acid. This can be used to oxidise a wide variety of biological molecules including amines, amino acids and thiols. It is generally considered the reactive oxidants produced by neutrophils cannot selectively differentiate between nearby targets, or act as specific toxins. For this reason neutrophil derived oxidative damage has been implicated in inflammatory diseases e.g. rheumatoid arthritis. However two properties of MPO many facilitate the degradation of specific regions of tissue. Firstly it is a highly charged protein, allowing it to bind directly to host tissues (Klebanoff 1988); secondly the high reactivity of HOCl may ensure that its' toxicity will be rapidly dissipated within a short distance of its' site of generation *in vivo* (Weiss 1989). Thus neutrophils may provide a very precise and effective mechanism to oxidise specific regions of tissue.

Only part of the destructive potential of neutrophils is provided by free radicals and proteinases also have an important role (Stadtman 1990). To outline the interactions between free radicals and proteinases in

tissue destruction is beyond the scope of this thesis but has been reviewed in some detail by Weiss (1989). In essence the oxidation induced inactivation of proteinase inhibitors and activation of proteinases can create an environment in which the destructive potential of oxidants is maximised.

## 1.0.4 Mitochondrial formation of reactive oxygen species

Most cellular process directly or indirectly utilise ATP, which is continuously regenerated through various metabolic pathways. The most efficient require oxidation of carbohydrate and fat. The final step of the oxidative process requires molecular oxygen as an electron acceptor and occurs in the Krebs cycle and the mitochondrial electron transport chain.

In biological systems the reduction of molecular oxygen ($O_2$) to water requires 4 electrons and occurs *via* 2 biochemical pathways. The main route is the tetravalent reduction of oxygen to water in the mitochondria, using cytochrome oxidase as the final catalyst. No oxygen intermediates are produced in this pathway, which has been estimated to account for 95 to 98% of the total oxygen consumption. The remaining oxygen is thought to be utilised in univalent reduction, resulting in the formation of highly reactive oxygen intermediates (Equ. 9). These reactive oxygen species include $O_2^{\bullet -}$ (a), which can be further reduced to $H_2O_2$ (b), $OH^{\bullet}$(c) and finally water (d).

$$\underset{}{O_2} \xrightarrow{e^-} \underset{(a)}{O_2^{\bullet -}} \xrightarrow{e^- + 2H^+} \underset{(b)}{H_2O_2} \xrightarrow{e^- + H^+} \underset{(c)}{OH^{\bullet}} \xrightarrow{e^- + H^+} \underset{(d)}{H_2O} \qquad \text{(Equ. 9)}$$

$$H_2O_2 \searrow H_2O$$

Univalent reduction of oxygen

The production of superoxide anions in mitochondrial membranes was first detected by Loschen *et al.* (1974), although the precise sites and

mechanisms for univalent reduction have not been elucidated. Mitochondria appear to be particularly effective in preventing the formation of reactive oxygen metabolites. It has been reported they consume 90% of cellular oxygen, yet produce only 15% of the reactive oxygen species in mammals (Asayama & Kato 1990). These authors proposed the remainder were produced by subcellular sites, including microsomes, peroxisomes, cytosol and nuclear membranes; however these claims were not substantiated with experimental data. Although the proposals of Asayama & Kato (1990) may accurately reflect free radical formation at rest, it is likely this relationship is perturbed during exercise. In this situation mitochondria are considered to be the main cellular source of reactive oxygen species (Sjodin *et al.* 1990, Witt *et al.* 1992).

### 1.0.5 The cellular basis of peroxidative damage

There is a common belief that all radicals are highly reactive and produce cellular damage, especially within the popular exercise literature (e.g. Colgan 1993, Pidcock 1995). Much of the notoriety of free radical reactivity relates to the hydroxyl radical, which rarely diffuses more than 1 or 2 molecular diameters before reacting with whatever is in close proximity (Pryor 1986). In contrast other oxygen centered radicals e.g. the superoxide radical are far less reactive, and are able to pass across biological membranes (Mao & Poznansky 1992). Many antioxidants e.g. ascorbate or tocopherol can reduce cell damage by themselves forming radicals (Equ.s 10 and 11). As these radical species are more stable, undesired oxidation of cell constituents can be minimised.

Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated fatty acids (Tappel 1973). This event (or more likely cycle of events), is initiated by the abstraction a hydrogen atom from a methylene carbon on a fatty acid, or fatty acyl side chain. The ease at which the hydrogen atom is removed is proportional to the number of double bonds in the fatty acid. Removal of a hydrogen atom leaves an unpaired electron on the carbon atom, to which it was originally attached. The most likely fate for the new compound is molecular rearrangement, followed by reaction with $O_2$ to give a peroxyl radical. In turn, this radical can damage membrane proteins, or abstract hydrogen from

adjacent fatty acids, thereby propagating lipid peroxidation. The ratio of unsaturated fatty acids to antioxidant compounds (including proteins) is related to the number of peroxidative cycles which occur in a membrane (Halliwell & Chirico 1993).

Membrane peroxidation is associated with structural and functional changes including fluidity (Tiidus & Houston 1995), permeability (Halliwell & Chirico 1993) and transporter function (De Groot *et al.* 1985, Robblee & Clandinin 1984). Kramer *et al.* (1984) demonstrated a direct correlation between membrane oxidation and enzyme inhibition, although the mechanism of inactivation was unclear. Proteins with aromatic or sulphur-containing amino acids may be susceptible to free radical induced amino acid modification (Stadtman 1990). The formation of cross links with peroxidation products such as malondialdehyde may also contribute to reduced membrane transporter activity (Kramer *et al.* 1984, Stadtman 1990). Thus the lipid microenvironments of transport proteins may influence the susceptibility of these ion channels to free radical mediated inactivation. These events are shown schematically in Fig. 1.1.

Traumatic or ischaemic injury is unusual, as it may produce tissue damage directly, by accelerating the peroxidation of cell membrane lipids (Halliwell & Chirico 1993). There are a vast number of chemical pathways through which free radicals can react with lipids and proteins, a few mechanisms are outlined in Fig. 1.1. Although oxidation of DNA could also threaten cell viability (Ames 1989), in the short term, the functional changes which result from lipid and protein oxidation are probably the greatest threat to cell survival.

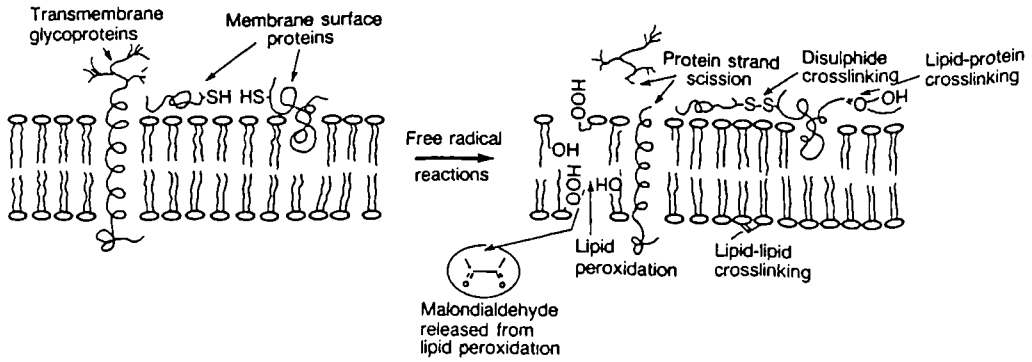


**Fig. 1.1 Free radical reactions with different membrane constituents.**

Reprinted from Sjodin *et al.* (1990)

## 1.0.6 Antioxidants

The term antioxidant can be applied to compounds which reduce oxidation. In biological systems three major groups of antioxidants are recognised 1) preventative, 2) sacrificial and 3) enzymatic.

Metal ion chelaters are considered the 'first line of defence' against unwanted oxidation. In the extracellular fluids proteins such as transferrin, caeruloplasmin and albumin bind transition metals (Maxwell *et al.* 1993), thereby reducing free radical formation *via* Fenton and Haber-Weiss reactions. Once free radicals have been formed these proteins are ineffective in protecting against oxidative damage, this function is performed by small reducing molecules (quenchers).

Sacrificial molecules or quenchers react with free radicals to form more stable compounds. This limits the propagation of free radical chain reactions, thereby minimising damage to more important biological molecules. Ascorbate has been described as an outstanding antioxidant in human blood plasma (Frei *et al.* 1989). In combination with uric acid it provides much of the protection against free radical damage outside the cell (Ames 1989). Reduced glutathione is highly concentrated within cells where typically its concentration exceeds 1 $mmol.l^{-1}$ (Allen 1991), although this is highly tissue dependent (Sen 1995). Within muscle reduced gutathione, carnitine and phenylalanine are highly concentrated (Kretzschmar & Muller 1993, Cooper *et al.* 1986, Ji 1995), where they are involved in the interception of free radicals. In cellular membranes this role is thought to be performed by vitamin E and β-carotene, which may help to limit membrane peroxidation.

Vitamin E is the collective name given to 8 naturally occurring compounds known as tocopherols and tocotrienols. These consist of a chromanol head and an isopreniod side chain, which is saturated in tocopherols and unsaturated tocotrienols (Kayden & Traber 1993). Of these lipid soluble chain breaking antioxidants, α-tocopherol is thought to be the most biologically active in lipid membranes. Typically one molecule of vitamin E is present per 1-2,000 fatty acids (Tappel 1980, Packer 1992), where it produces several beneficial effects regarding membrane function. Membrane microviscosity appears to be increased, while passive permeability to low molecular weight substances is decreased (Tiidus & Houston 1995). These effects are thought to occur *via* interactions between methyl groups, on the isoprenoid side chains and

the *cis*-double bonds of the polyunsaturated membrane phospholipids; which increase the 'orderliness of membrane lipid packing' (Tiidus & Houston 1995). The antioxidant properties of vitamin E may offer protection against membrane peroxidation.

It is recognised that antioxidants often act synergistically to regenerate other antioxidants. For example the loss or consumption of vitamin E is thought to be prevented by Vitamin C (Packer 1992), although this has not been demonstrated *in vivo* (Liebler 1993). A schematic representation of the termination of lipid peroxidation by abstraction of hydrogen from tocopherol is shown in Equ. 10; where $LO_2^{\bullet}$ represents a peroxyl radical. The subsequent regeneration of tocopherol by aqueous ascorbate is shown in Equ. 11, while the regeneration of ascorbate is facilitated by dihydrolipate and reduced glutathione (Packer 1992).

$$\text{Tocopherol} + LO_2^{\bullet} \Rightarrow LO_2H + \text{Tocopherol}^{\bullet} \qquad \text{(Equ. 10)}$$

$$\text{Tocopherol}^{\bullet} + \text{Ascorbate} \Rightarrow \text{Tocopherol} + \text{Ascorbate}^{\bullet} \qquad \text{(Equ. 11)}$$

In addition to the redox cycling of aqueous antioxidants there are also interactions with enzymatic antioxidant systems to enhance protection against oxidative damage (Packer 1992). A schematic representation is shown in Fig. 1.2. In biological systems antioxidant compounds such as ascorbate, tocopherol and glutathione are sometimes referred to as 'low molecular weight' antioxidants to distinguish between sacrificial and enzymatic antioxidants.

Enzymatic antioxidants have relatively low activity in extracellular fluids, where much of the antioxidant protection is provided by the sacrificial molecules. Within the cell however enzymatic antioxidants play a more important role in the free radical defence system, where they catalyse reactions which reduce the concentration of reactive oxygen species (without directly reacting with their specific substrates). Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase are the three major antioxidant enzymes in mammals. All act in solution and have metal co-factors at their active sites. SOD requires Fe, Mn or Cu, catalase Fe, and GPx selenium. Their reactions are summarised overleaf.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H+ \Rightarrow H_2O_2 + O_2 \qquad \text{SOD} \qquad \text{(Equ. 12)}$$

$$2H_2O_2 \Rightarrow O_2 + 2H_2O \qquad \text{Catalase} \qquad \text{(Equ. 13)}$$

$$ROOH + 2GSH \Rightarrow ROH + H_2O + GSSG \quad \text{GPx} \qquad \text{(Equ. 14)}$$

*Glutathione reductase*

NADP+ NADPH + H+

The spontaneous or catalysed dismutation of $O_2^{\bullet-}$ by SOD generates $H_2O_2$, which is a harmful oxidant in cells. Thus, to respond to an increase in $O_2^{\bullet-}$ formation cells must also increase activity of SOD, GPx and catalase to regulate $H_2O_2$. Without appropriate co-ordination of antioxidant enzyme expression, oxidative damage can also result (Nicotera *et al.* 1989, Harris 1992). Antioxidant enzymes are regulated *via* a variety of mechanisms, for example IL-1 induces SOD, while *oxy*R is induced by $H_2O_2$ and in turn induces both catalase and glutathione reductase (Harris 1992). A schematic representation of cellular antioxidant defences shown in Fig. 1.2.

Many compounds exhibit qualities which reduce oxidation, and are commonly described as antioxidants. The action of such compounds is however dependent upon their micro environment. Under several *in vitro* conditions antioxidants can promote oxidation. For example, both vitamins E and C act as pro-oxidants in the presence of free iron (Yamamoto & Niki 1988). Cu/Zn SOD can catalyse $OH^{\bullet}$ radical formation from $H_2O_2$, the product of the enzymes' dismutation of $O_2^{\bullet-}$ (Yim *et al.* 1991).

In conditions associated with increased free radical formation *in vivo*, some studies suggest antioxidant supplementation can produce detrimental effects. Increased plasma creatine kinase activity (CK) has been reported in exercised animals supplemented with vitamin E (Efremov & Sakaeva 1974). In patients with rheumatoid arthritis dietary supplementation with vitamins C and E produced detrimental changes in about 40% of patients (Lunec 1996). Although these cannot be regarded as typical responses, such studies demonstrate antioxidant administration may not always be beneficial in all conditions associated with the overproduction of free radicals.

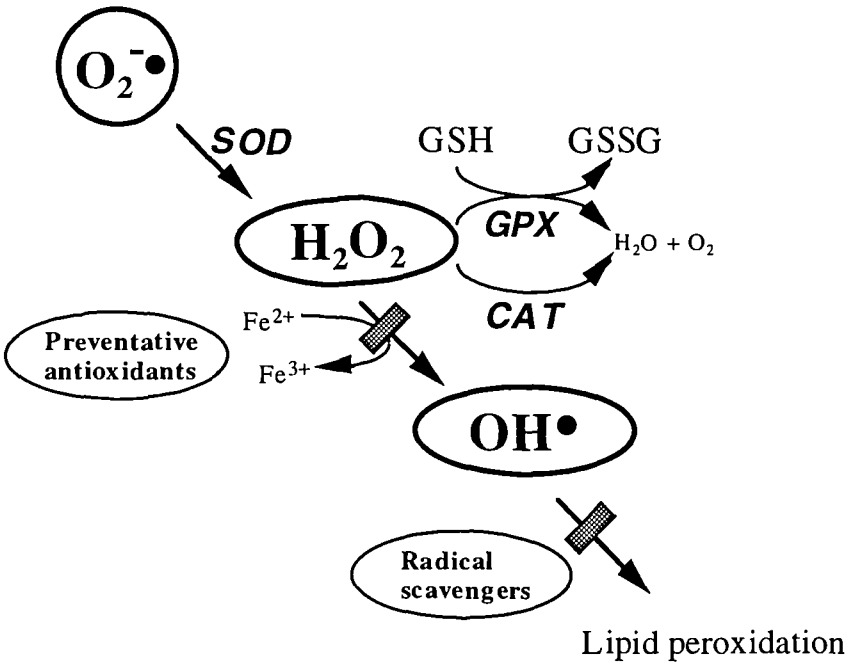


**Fig. 1.2 Defence mechanisms against damage by reactive oxygen species.**

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) eliminate many potentially damaging oxygen species. Preventative antioxidants (e.g. carnosine) bind transition metals e.g. $Fe^{2+}$ and $Cu^{+}$. Radical scavengers such as vitamin E further limit damage. Grey bands indicate points at which free radical formation and/or propagation are limited. Reactive oxygen species, $O_2^{\bullet-}$ (Superoxide radical), $H_2O_2$ (Hydrogen peroxide), hydroxyl radical ($OH^\bullet$). GSH (reduced glutathione), GSSG (oxidised glutathione).

### 1.0.7 Repair of oxidative damage

Cellular function is facilitated by membrane proteins and lipids which may be compromised when these structures are oxidised by free radicals (Kramer *et al.* 1984, Sen *et al.* 1995). A number of systems exist to remove and repair oxidised DNA, proteins and lipids.

Oxidative damage to proteins has been linked with several pathological processes associated with increased free radical production (Stadtman 1990). When oxidised, proteins are more susceptible to breakdown by calpains and proteinases. It appears inflammatory cells (e.g. neutrophils) may use oxidative modification to 'mark' specific proteins for degradation (Weiss 1989, Stadtman 1990). It is believed lysosomal proteases are responsible for the degradation of cellular organelles, while cytosolic proteinases, proteases and peptidases are responsible for the degradation of cytoplasmic proteins (Davies 1993). The resultant removal of oxidatively damaged structures has been considered as the first step of the repair process (Davies 1993).

Exercise has also been demonstrated to increase the protein carbonyl content of rodent (Witt *et al.* 1992) and human muscle (Saxton *et al.* 1994). In human muscle, exercise induced increases in protein carbonyls may return to normal levels within a day of exercise (Saxton *et al.* 1994), suggesting such compounds are cleared relatively quickly. Proteins oxidised during exercise may be repaired *via* similar mechanisms to oxidatively labeled proteins.

Oxidised membrane phospholipids are preferentially degraded by phospholipase $A_2$ (van Kuijk *et al.* 1987), which may be considered as the first step in membrane repair. Phospholipase $A_2$ appears essential for the reduction of membrane phospholipid hydroperoxides using glutathione (van Kuijk *et al.* 1987). The removal of such compounds could limit peroxidative chain reactions and the formation of reactive products such as aldehydes (van Kuijk *et al.* 1987). The final sequence of events in the membrane repair process is thought to involve the re-acylation of the lysophopholipid (produced by the removal of the hydroperoxide), with a long chain fatty acyl CoA (van Kuijk *et al.* 1987).

Thus, the mammalian antioxidant defence system is complemented by sophisticated mechanisms to repair oxidatively damaged structures. The existence of such highly specialised systems suggests both the formation of free radicals, and oxidative damage, are normal consequences of aerobic metabolism.

1.0.8 Detection and measurement of free radicals

The highly reactive and short-lived nature of many radicals makes them extremely difficult to detect and quantify (Junod 1989). The only direct method to detect free radicals is Electron Paramagnetic Resonance (EPR), with or without spin trapping (Aust et al 1993). Such techniques have not been used extensively to assess changes in radical signals in association with exercise. Davies *et al.* (1982) has reported an increase in radical signals in rodent liver and muscle homogenates following exercise. Jackson *et al.* (1985) demonstrated a 70% increase in the Electron Spin Resonance (ESR) signal from electrically stimulated rat muscle. These studies provide direct evidence that exercise can increase the formation of free radicals.

Practical (Das *et al.* 1989) and technical limitations to EPR (Powell & Hall 1990) force most researchers to adopt indirect markers to assess free radical activity. These can be roughly subdivided into two groups, markers of free radical stress and markers of free radical damage. Although these terms have been used interchangeably in the exercise literature, it is important to distinguish between stress and damage markers as they influence the interpretation of experimental results.

Changes in damage markers may demonstrate a saturation of cell antioxidant mechanisms such that important cell components are attacked. Damage markers in tissue may include oxidised lipids (Halliwell & Chirico 1993), proteins (Levine *et al.* 1990), and nucleic acids (Ames 1989, Fraga *et al.* 1990). Products of oxidative damage may also be detected in blood e.g. malondialdehyde (MDA) and urine e.g. 8-hydroxy-2'-deoxyguanosine (Fraga *et al.* 1990). In expired air low molecular weight hydrocarbons (Wade & van Rij 1985, Kanter *et al.* 1993) and spontaneous chemiluminesence (Williams & Chance 1982) have also been used as indices of lipid peroxidation in man.

Tiidus & Houston (1995) defined oxidative stress as "an elevation in the steady state concentration of free radicals due to an imbalance in free radical generation versus cellular antioxidant defenses." This definition is untenable as it is currently impossible to measure the concentration of free radicals in a biological system. Furthermore, changes which occur during exercise may simultaneously alter both intracellular antioxidant defences (Kretschzmar & Muller 1993, Sen 1995) and free radical production by cellular organelles e.g.

mitochondria. This situation is further complicated by intra and inter tissue differences in the relative free radical protection provided by enzymatic and chain breaking antioxidants (Mbemba *et al.* 1985, Sen 1995).

To circumvent the problems of free radical and antioxidant measurement, free radical stress/oxidative stress will be defined as:-

> "..... a state in which exposure to free radicals or other oxidants represents a challenge to normal function."
>
> Davies (1993)

Sacrificial antioxidants preferentially combine with radicals to minimise damage to more important cellular constituents. Such properties may allow them to be used as markers of markers of oxidative stress, although interpretation of *in vivo* changes is often complicated by intercompartmental transport (Kretzschmar & Muller 1993, Duarte *et al.* 1994). Stress markers may provide a more sensitive measure of increased radical formation, as their redox state and /or concentration may be altered before saturation of antioxidant defences. Conversely radical damage markers are less sensitive to changes in oxidative stress, but by definition show an overpowering of antioxidant systems.

### 1.0.9 Exercise models to induce free radical stress

In man, whole body oxygen consumption can rise 20 fold during intense exercise (Brooks & Fahey 1984), although the oxygen flux in individual muscle fibres is thought to rise 1-200 fold (Keul *et al.* 1972). *In vitro* experiments suggest similar increases occur in free radical production and thus free radical stress. There is evidence that free radical production rises exponentially rather than linearly, as a consequence of increasing exercise intensity (Diaz *et al.* 1993, Nashawati *et al.* 1993); although more detailed studies are needed.

Exercise is also likely to increase the production of radicals *via* the uptake of airborne pollutants (Cross *et al.* 1992) and mechanical process (Symons 1988). Although these are minor sources of free radicals at the

whole body level, they may still produce damage at, or close to, their sites of formation. In summary, using the rise in $VO_2$ during exercise to estimate the relative increase in free radical stress, probably underestimates the true oxidative stress incurred during physical activity.

The production of free radicals as a consequence of muscular work is dependent upon the type of muscle contraction utilised during the exercise. The metabolic cost of concentric muscular work has been estimated to be as much as seven times that of eccentric exercise (Asmussen 1953). The difference in $VO_2$ between exercise modalities is augmented as the intensity of exercise increases (Evans & Cannon 1991). The greater metabolic and circulatory demands imposed by concentric work may have the potential to produce ischaemic reperfusion injury, which is discussed in section 1.0.10.

Metabolic and hyperthemic mechanisms of initial muscle injury have received little attention in relation to eccentric exercise, as such activity are thought to be 'less stressful' than similar concentric exercise (Byrnes & Clarkson 1986). Nadel *et al.* (1972) reported a higher relative muscle temperature when comparing eccentric and concentric actions. The authors speculated that this effect might be a consequence of lower muscle blood flow during eccentric actions. Fibre recruitment patterns are also thought to be modified during eccentric actions (Newham *et al.* 1983c, Nardone *et al.* 1989), such that fewer fibres are activated. Such fibres would clearly have a higher oxygen uptake than would be predicted from whole body $VO_2$ measurements and may also be at a higher temperature than during similar concentric work. Therefore metabolic and thermal damage cannot be discounted as sources of initial injury during eccentric actions. As even the most intense high force eccentric exercise imposes modest whole body metabolic and circulatory demands, ischaemic reperfusion injury would appear to be an unlikely consequence of such activity.

Ultrastructural damage produced by eccentric actions is well documented (Friden *et al.* 1983, Stauber *et al.* 1990), which may result in an inflammatory response within muscle fibres and connective tissue (Jones *et al.* 1986, Round *et al.* 1987). A number of inflammatory processes may produce elevations in free radical stress several days after eccentric exercise. The most commonly recognised is the production of hypochlorous acid by activated neutrophils (Babior 1984, Weiss 1989). It has also been proposed the production of reactive oxygen species and

release of proteases may compromise the binding of transition metals (Kanter 1995), thereby allowing their participation in pro-oxidant reactions. However the co-incident release of lactoferrin appears to prevent free radical formation *via* such mechanisms (Weiss 1989).

Exercises involving eccentric or concentric actions exclusively, possibly provide the most useful experimental models to investigate the role of free radicals in exercise induced muscle damage. At the whole body level eccentric muscle actions may impose minimal oxidative stress during exercise, but may result in free radical stress in association with tissue repair. Conversely concentric contractions offer a mechanism to impose maximal muscular oxidative stress, without mechanical injury (Gohil *et al.* 1988). For this reason concentric and eccentric muscle actions can be used to separate the effects of free radical stress occurring during exercise, from that associated with inflammation.

No physical activity encountered in daily life utilises one type of muscular contraction exclusively. Exercise such as running has often been used to investigate exercise induced muscle damage, especially in experimental animals (Salminen & Kihlstrom 1985, Duarte *et al.* 1994). Such exercise imposes high metabolic and circulatory demands but also involves high force eccentric contractions. Running is known to produce myofibrilar disruption in man (Hikida *et al.* 1983) and rodents (Salminen & Kihlstrom 1985). The combined metabolic stress, muscle damage and possibility of ischaemic injury to non muscle tissues (Davies *et al.* 1982), can make the sources of free radicals difficult to elucidate following such activities. This can complicate the interpretation of changes in markers of free radical stress or damage, especially if these are assessed in blood or urine.

When using experimental animals, free radical damage indices in muscle can be assessed more frequently than is ethically viable in humans. There are however several limitations with animal experimentation. One of the most important is species differences, which under some circumstances may raise questions of validity when extrapolating experimental findings to man. For example, rodents can synthesise ascorbate, which may question their use as models to investigate conditions associated with increased free radical stress e.g. inflammation or exercise. Guinea pigs overcome the problem of ascorbate synthesis but do not run spontaneously (Witt *et al.* 1992).

Following muscle damaging exercise cellular infiltration is often observed in the damaged tissue. Differences exist in the time course of inflammatory events between humans and rodents (Jones *et al.* 1986, Komulainen & Vihko 1994), which have been acknowledged previously (Smith 1991). There is also a lack homology between the properties of human neutrophils and neutrophils from animals commonly used in experimental models. For example, neutrophils from rats contain small amounts of elastase (Virca *et al.* 1984), while rabbit neutrophils rely predominantly on acid rather than neutral proteases (Sinha *et al.* 1988). Although these differences may appear trivial they are linked to important variations in their antiproteinase screens, which are intimately related to oxidation mediated inactivation (Weiss 1989). Thus some free radical mediated events appear to differ between rodents and humans, Weiss (1989) proposed caution must be used when extending to humans, insights obtained in animals.

Despite these differences rodents are still the most popular choice for studies on free radicals and exercise (Zerba *et al.* 1990, Warren *et al.* 1992, Duarte *et al.* 1994). Although there are limitations, such investigations have helped to establish models of exercise induced muscle damage (e.g. Armstrong *et al.* 1991) and inflammatory processes (e.g. Smith 1991, MacIntyre *et al.* 1995).

### 1.0.10 Possible roles of free radicals in exercise induced muscle damage

Witt *et al.* (1992) proposed a theoretical model linking exercise with increased free radical production both during and following exercise. These authors speculated that the decrease in blood flow to organs and tissues (e.g. kidney and splancnic region) could result in hypoxia; while muscle could also become hypoxic close to $VO_2$ max. At the cessation of exercise the return of re-oxygenated blood to these regions may lead to 'respiratory burst'. Exercise induced ischaemia is more likely to occur when performing prolonged whole body exercise in hot environments, as such activity imposes substantial metabolic and circulatory demands.

In experimental reperfusion models, free radical mediated injury may result from the uncoupling of mitochondrial respiratory control (Gauduel *et al.* 1989) and activation of xanthine oxidase (McCord 1985,

Lazzarino *et al.* 1994). The xanthine oxidase system is also thought to produce free radical stress in fatigued muscle (Diaz *et al.* 1993, Grisham & Granger 1989, Sjodin *et al.* 1990), although unlike ischaemia, insufficiency in ATP regeneration is not due to lack of oxygen (Stainsby *et al.* 1989), but the high rate of ATP turnover (Sjodin *et al.* 1990).

Although exercise induced reperfusion injury is a popular concept (e.g. Armstrong 1986, Singh 1992, Jakeman & Maxwell 1993), it appears unlikely skeletal muscle could be damaged by such mechanisms. Subjects can rarely exercise for more than 2-3 minutes at $VO_2$ max, thus if muscle ischaemia occurs it is likely to be extremely transitory. Mair *et al.* (1995) found no evidence of free radical mediated damage following cardiac surgery and concluded human striated muscle is extremely resistant to reperfusion injury. During such procedures the severity and duration of occlusion is likely to be far greater than encountered by skeletal muscle during exercise, thus the possibility of exercise induced reperfusion injury appears remote.

Free radicals also appear to be obligatory for optimal muscle contractility (Reid *et al.* 1993). However there is also evidence linking free radical stress in non-ischaemic muscle with reduced contractility (Barclay & Hansel 1990, Reid *et al.* 1992, Nashawati *et al.* 1993). Under such conditions reductions in force might arise from membrane damage to the sarcoplasmic reticulum (SR) and/or sarcolemma. Nashawati *et al.* (1993) observed a marked decline in twitch tension after radical stress in dogs and suggested damage to cellular structures (e.g. intracellular membranes) may have been responsible for alterations in contractile function. Such proposals are consistent with reduced $Na^+/K^+$ pump activity in peroxisomal and sarcolemmal membranes following peroxidation (Kramer *et al.* 1984). Similarly Sen *et al.* (1995) demonstrated potassium transport systems were extremely sensitive to oxidant exposure. Sarcolemmal peroxidation is known result in $K^+$ efflux (Sugihara *et al.* 1991) and Knochel (1982) has reported hyperkalaemia (elevated blood $K^+$) in association with rhabdomyolysis. Sarcolemmal damage might alter the ionic environment around damaged fibres, thereby modifying action potential propagation; while damage to the SR could modify calcium release. The intracellular and extracellular changes which are likely to result from such damage could clearly modify contractile function. Paradoxically free radical mediated muscle damage

may have the potential to compromise force production by the same processes which reduce muscle contractility during fatigue.

Changes in muscle contractility are also observed following fatiguing exercise (Edwards *et al.* 1977, Fitts 1994), although if the exercise does not result in muscle damage these changes are quickly reversed. Free radical mediated damage to the structures responsible for myocellular homeostasis might prolong fatigue following exercise. Furthermore, delayed oxidative damage to, and impaired functioning of homeostatic mechanisms might also produce delayed changes in contractile function. Increased permeability to $Ca^{2+}$ is one of the best documented and potentially damaging effects associated with membrane peroxidation (Halliwell & Chirico 1993, Wu *et al.* 1996). Such damage may allow the cytosolic calcium concentration to rise to sufficiently high levels to activate a spectrum of proteases (Dayton *et al.* 1979, Weiss 1989). In muscle Z-discs, troponin and tropomyosin appear to be particularly sensitive to calpain mediated proteolytic degradation (Busch *et al.* 1972, Dayton *et al.* 1976, Cullen & Fulthorpe 1982). This has been termed the autogenic phase of muscle damage (Armstrong *et al.* 1991) and such events are thought to contribute to cell necrosis in damaged muscle (Jackson *et al.* 1984, Jackson 1990).

A reduction in the force produced by skeletal muscle when applying low frequency stimulation (LFF) is characteristic of muscle damage. Using *in vitro* techniques Reid *et al.* (1992) demonstrated antioxidants could attenuate LFF in rat diaphragm; there is also evidence that similar effects can be reproduced in human muscle *in vivo*. Jakeman & Maxwell (1993) reported vitamin C supplementation attenuated LFF following damaging eccentric exercise. These authors discussed the possibility that vitamin C may have produced this effect by reducing oxidative damage to the $Na^+/K^+$ pumps of the SR. However when the same study was reported with additional biochemical data (Maxwell *et al.* 1993), the plasma MDA measurements indicated vitamin supplementation produced pro-oxidant effects. Relative to the control group plasma MDA was elevated 10% and 41% pre-exercise in vitamin C and E supplemented groups respectively. Following exercise MDA was elevated 20% and 89% in vitamin C and E supplemented groups respectively.

The paradox of improved muscle functional accompanied by increased lipid peroxidation with vitamin C supplementation is difficult

to resolve. It may indicate the 'secondary' effects of vitamin C supplementation (Section 1.0.6) may be more important in attenuating muscle damage than its potential role as an antioxidant. In addition to its antioxidant role, vitamin E is also known to have membrane stabilising effects on lysosomes (Fong *et al.* 1973) and neutophils (Luostarinen *et al.* 1991). This could potentially reduce the release of proteases (Fong *et al.* 1973), thereby minimising tissue injury mediated by such mechanisms. Vitamin E is also thought to reduce the chemotaxis of human neutrophils (Luostarinen *et al.* 1991). Such antinflammatory effects of antioxidants might also reduce tissue injury (Weiss 1989). Although vitamin E supplementation can reduce protein oxidation during exercise (Reznick *et al.* 1992) this intervention appears to be ineffective against mechanically induced damage (Warren *et al.* 1992, Jakeman & Maxwell 1993).

Perhaps the most convincing evidence implicating free radical involvement in muscle damage and force loss was documented by Zerba *et al.* (1990), in which controlled lengthening contractions of the extensor *digitorum longus* were used to produce muscle injury. Intraperitoneal injections of SOD were given to young, adult and old mice 24 hrs pre-injury and at 24 and 48 hrs post-injury. In old mice SOD attenuated the decline in peak tension 10 mins post-exercise and reduced the loss of peak tension in all age groups 3 days after eccentric contractions. Such observations implicate the superoxide radical in both fatigue and delayed force loss, Armstrong *et al.* (1991) and Warren *et al.* (1992) proposed the latter event may be related to phagocytic activity.

### 1.0.11 Production of muscle damage using eccentric muscle actions

Three types of muscle action are recognised, these are isometric, during which the muscle generates force without altering its length; concentric, during which the muscle shortens and eccentric, during which the muscle is forcibly lengthened. There is however some debate regarding the terminology for eccentric muscle actions (Stauber 1989) and such movements have also been described as lengthening contractions (McCully & Faulkner 1986) and pliometric actions (Knuttgen & Kraemer 1987). The type of muscle action and velocity of muscle length change, have profound effects on the muscle force developed.

The maximal muscle force developed concentrically at a given length varies inversely with the velocity of movement (i.e. the faster the movement the lower the maximal force produced). In contrast, eccentric actions develop greater force at faster velocities of lengthening, within a certain range of velocities. The maximum force developed during eccentric actions is about 1.8 times greater than the maximum isometric force, which in turn is always greater than the maximal concentric force. These muscle properties are illustrated in Fig. 1.3 . Although this data was originally obtained for rodent muscle *in vitro*, the human quadriceps also appear to have similar *in vivo* properties (Westing *et al.* 1988).

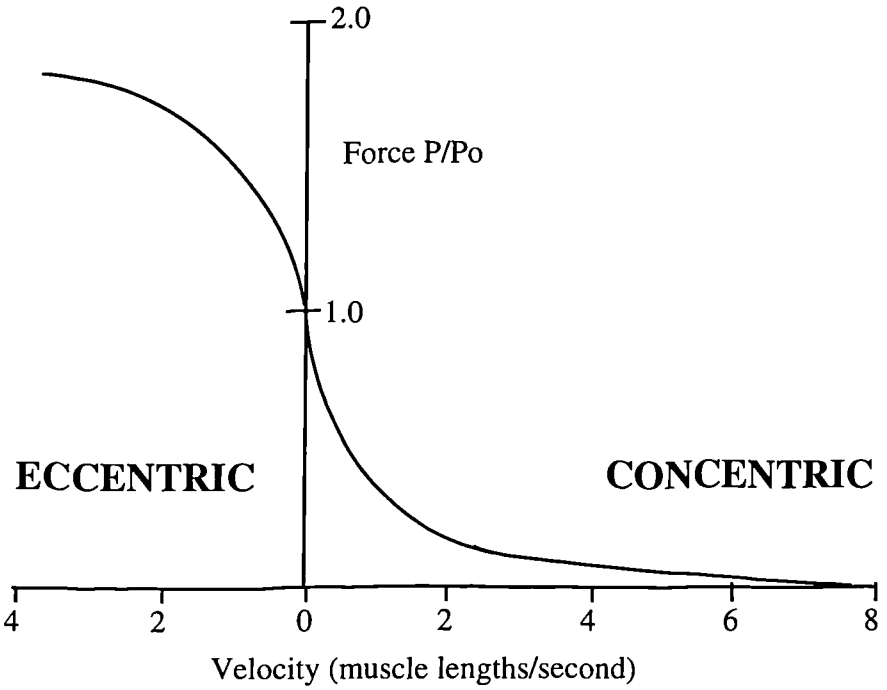


**Fig. 1.3 Force velocity curve for mouse soleus during eccentric and concentric actions at 26°C, where Po represents the peak isometric force.**

(Redrawn from Jones & Round 1993).

Under comparable conditions (i.e. duration, intensity and number of actions performed) eccentric actions are considerably more damaging than isometric or concentric actions (Talag 1973, Newham *et al.* 1983b, Saxton *et al.* 1994).

The initial event in eccentric muscle injury appears to be mechanical in origin (McCully & Faulkner 1986, Armstrong *et al.* 1991, Friden & Lieber 1992, Warren *et al.* 1993), although the relative importance of the specific mechanical factors which produce fibre injury are unclear. The integrated electromyograph (IEMG) produced during eccentric and concentric actions resulting in similar force, show the IEMG is lower for eccentric actions (Aura & Komi 1986, Berry *et al.* 1990). Several researchers have speculated active muscle fibres generate greater tension during eccentric, compared to isometric or concentric muscle actions (e.g. Newham *et al.* 1983b, Berry *et al.* 1990). It has also been proposed high fibre tension produces muscle damage (Katz 1939, Friden *et al.* 1983, Armstrong *et al.* 1991). Fibre tension has been altered independently of other mechanical factors in rodent (McCully & Faulkner 1986, Warren *et al.* 1993) and rabbit muscle (Lieber & Friden 1993). Muscle injury can be associated with the generation of high muscle forces (McCully & Faulkner 1986), although this findings is equivocal (Brooks *et al.* 1995, Lieber & Friden 1993). At least two other mechanical factors are known to contribute to initial muscle injury. Strain rate, i.e. the rate of muscle lengthening and strain magnitude i.e. the increase in muscle length (usually expressed as a percentage of Po) have also been implicated in eccentric exercise induced muscle damage (Brooks *et al.* 1995, Lieber & Friden 1993). When strain magnitude is high, sarcomeres are extended (1.4b) and the force produced is comparatively low (Fig. 1.4a). Despite the comparatively low muscle force, susceptibility to muscle injury appears to be increased (Lieber & Friden 1993).

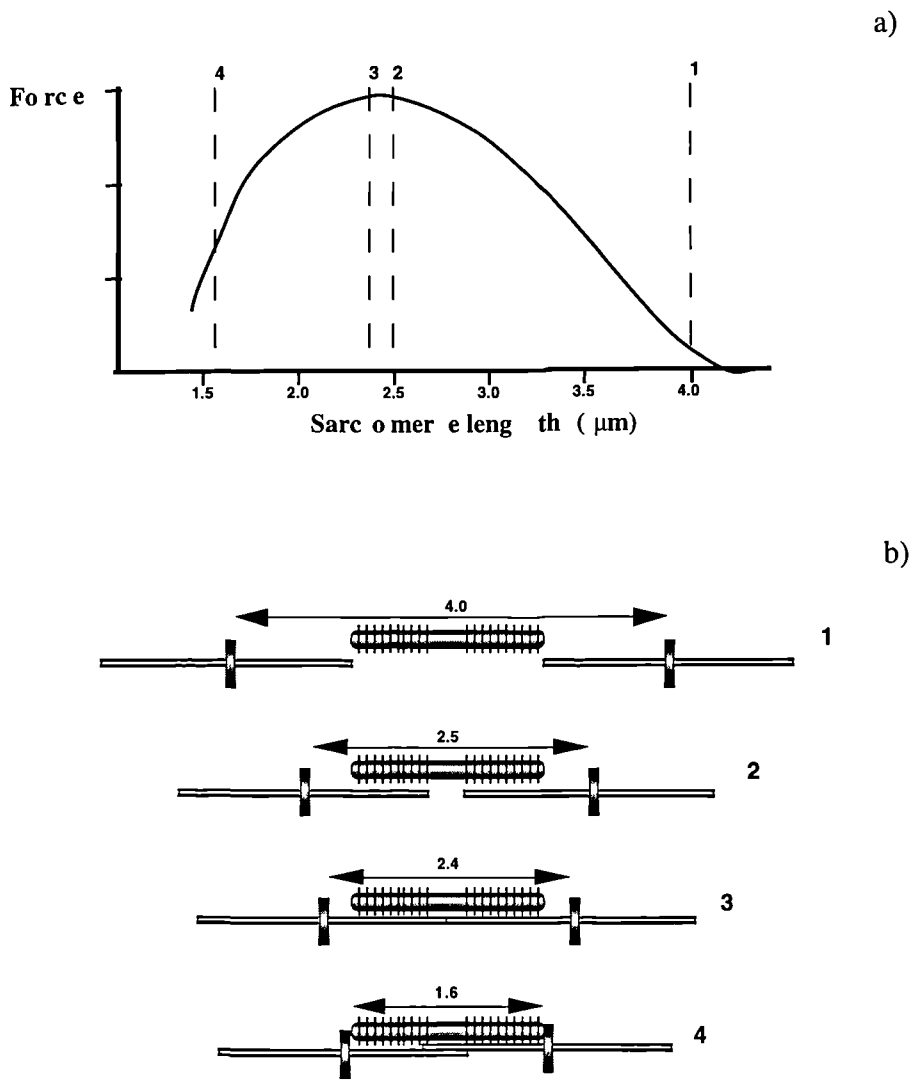


**Fig. 1.4 Isometric force at different sarcomere lengths. a) force generated, b) arrangement of filaments at different lengths.**

Redrawn from Gordon *et al.* (1966), with mammalian sarcomere lengths from Jones & Round (1993).

Initial muscle injury is closely associated with the mechanics of muscle contraction, however the cause for the delayed rise in muscle damage indices is poorly understood. Based on observations in animal experiments Armstrong *et al.* (1991) proposed a theoretical model to explain various aspects of the damage and regeneration process.

The initial phase is thought to begin with subcellular lesions in the fibres contractile and cytoskeletal components (Newham *et al.* 1983b; Friden *et al.* 1983; Armstrong *et al.* 1983). This may produce elevations in muscle calcium as a direct consequence of mechanical trauma or *via* free radical mediated mechanisms discussed in section (1.0.10). The loss of calcium homeostasis and subsequent activation of proteases may release chemoattractants (Armstrong *et al.* 1991), resulting in muscle inflammation. At a structural level these changes appear to co-incide with increased myofilament damage (Newham *et al.* 1983b, Friden *et al.* 1983). Ionic and structural changes resulting from muscle damage may contribute to LFF and isometric force decrements, which may persist for several days after the exercise bout (Clarkson *et al.* 1992).

Biochemical muscle damage indices such as the release of myocellular proteins suggest there is a delayed elevation in sarcolemmal permeability following exercise (Newham *et al.* 1986a, Jones *et al.* 1986). This may occur as a consequence of membrane peroxidation, described previously in Section 1.05. Muscle soreness (MS) is often associated with these events (Hough 1902), commencing approximately 12 hours post exercise and persisting for several days (Byrnes & Clarkson 1986, Clarkson *et al.* 1992).

A number of problems occur when using downhill running models to elucidate the mechanisms of exercise induced muscle and free radical damage. Some have briefly been discussed, such as high metabolic stress and reperfusion injury of organs (sections 1.0.9 and 1.0.10). Other problems arise due to the rapid adaptation of the exercised muscle (Clarkson & Tremblay 1988), making it difficult to use multi treatment designs where subjects act as their own controls. Box stepping tasks have been used successfully to produce muscle damage (Newham *et al.* 1986a, Jakeman & Maxwell 1993). Typically one leg works eccentrically and the other works concentrically throughout the exercise. Such activity produces considerable whole body metabolic demands, making the source(s) of free radical stress difficult to elucidate. The need to exercise for several hours to produce muscle damage may make such protocols impractical for research with large subject numbers.

## 1.1.0 Methods for detection of muscle damage

### 1.1.1 Isometric force measurement

Decrements in force production may arise from impairments to one or more of the processes outlined in Fig. 1.5 . Faulkner *et al.* (1993) concluded the decrease in the maximum force developed by a muscle provides the most valid measure of the totality of injury. Changes in the intramuscular concentration of protons, calcium and sodium may also contribute to the observed force deficit following activity (Edwards *et al.* 1977, Fitts 1994), especially if the exercise is prolonged or intense. In undamaged muscle these changes would be quickly reversed, however free radicals may have the potential to further compromise force production (Section 1.0.10).

It has also been proposed the impairment in maximum voluntary force generation following damaging exercise may be partially attributable to inhibitory mechanisms in the affected musculature (Talag 1973). Afferent mechanisms are known to exist, which may have the potential to modify motor output (Bigland-Ritchie *et al.* 1983a, Bigland-Ritchie *et al.* 1983b, Hayward *et al.* 1991). If muscle damage results in neural inhibition, voluntary force measurements may overestimate the functional impairment at the muscular level. Such central neural effects and peripheral functional deficits could potentially be separated using myostimulation.

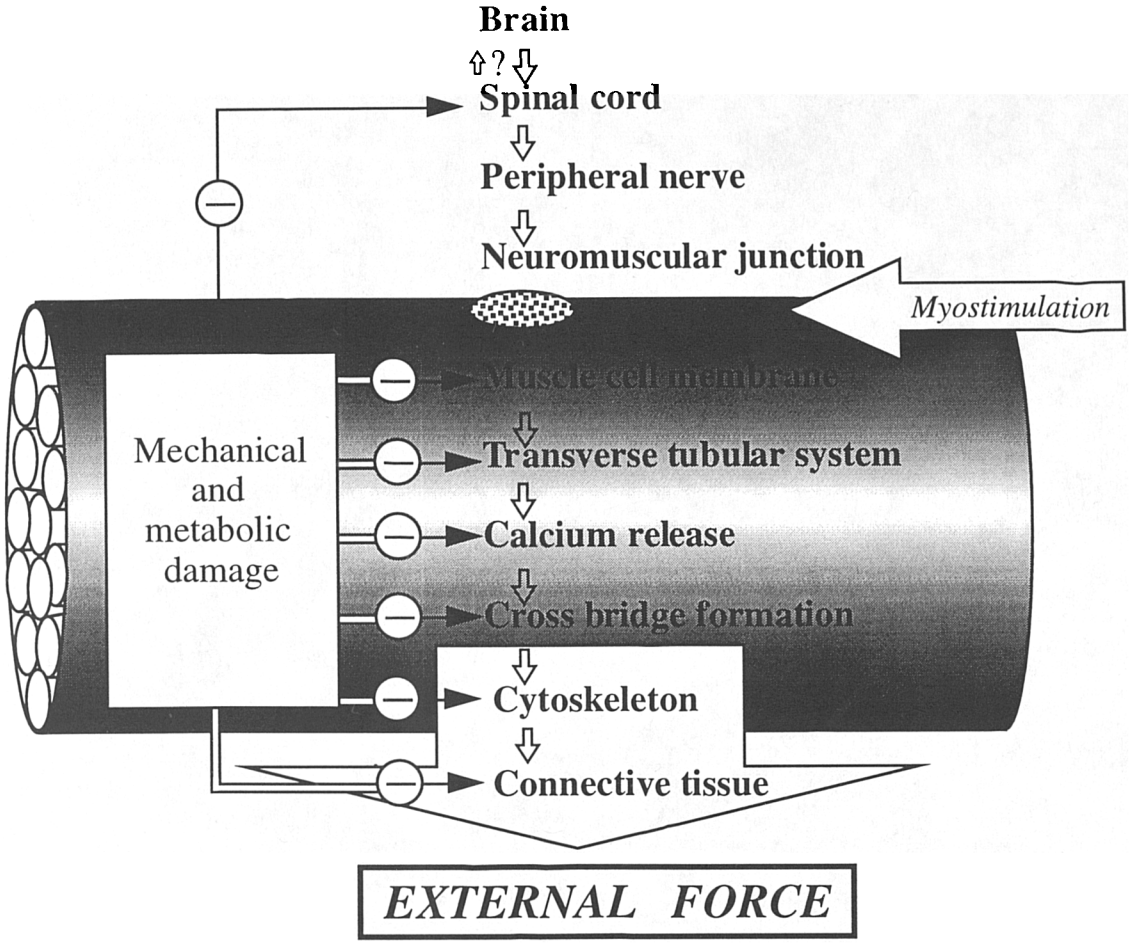


**Fig. 1.5 Processes involved in production of external force, with possible influences of exercise induced muscle damage.**

Negative symbols indicate metabolic or mechanical damage to specific structures which result in reduced external force.

## 1.1.2 Myostimulation

Functional deficits at the muscular level can be investigated using percutaneous electrical myostimulation (PES). Using this technique the chain of events resulting in muscular contraction starts with activation of the surface membrane (Lannergren & Westerblad 1989), either directly or

*via* the motor nerves. By superimposing PES on maximal voluntary contractions it is possible to determine if the voluntary force produced accurately reflects the force generating potential of the muscle. Therefore central limitations to maximum force production (i.e. those arising in the brain or spinal cord) can be detected.

Changes in peripheral muscle contractility such as LFF can be evaluated using PES to activate the muscle at low (20Hz) and high (100Hz) stimulation frequencies. The greater loss of force at low stimulation frequencies could be related to impairments in several of the processes outlined in Fig. 1.5; which might arise as a consequence of muscle damage. Jones & Round (1993) suggested LFF was caused by reduced affinity of troponin for calcium, inadequate calcium release or reduced T-tubule conductance. Decreased membrane excitability such that the applied stimulation frequency elicited fewer muscle action potentials could also explain the LFF phenomenon (Fitts 1994). At a structural level, low frequency fatigue might be related to sarcoplasmic reticulum damage, such that calcium release is impaired and/or damage to the sarcolemmal membrane, such that action potential propagation is reduced.

### 1.1.3. Serum creatine kinase activity (CK)

Creatine kinase catalyses the conversion of creatine phosphate to creatine and ATP. This pathway is extremely important for the generation of ATP during supermaximal exercise and high activity is detected in muscle. Isoforms of the enzyme are also found in brain tissue (Turner & Eppenberger 1973, Walliman *et al.* 1977). Within striated muscle creatine kinase activity is primarily detected in the cytosol and mitochondria, although evidence has also been presented that creatine kinase is bound to muscle myofibrils (Turner & Eppenberger 1973). Permeability changes to the sarcolemmal membrane allow this enzyme to exit the cell. Although rupture of the sarcolemma has been demonstrated following exercise in humans (Hikida *et al.* 1983), this may be an atypical route of enzyme release. More subtle changes within the sarcolemmal membrane such as lipid peroxidation are known to result in myocellular enzyme release (Gauduel *et al.* 1989, Lazzarino *et al.* 1994). Such observations

may be consistent with the proposals of Cannon *et al.* (1990) who suggested an exercise induced rise in CK may reflect membrane damage caused by activated neutrophils.

Creatine kinase has a molecular weight of 82kDa (Wilkinson 1970), which may prevent it from entering the blood directly when released by the muscle. It is believed creatine kinase enters the blood *via* the lymphatic system, which is thought to produce an appreciable delay between muscle release and appearance in blood (Volfinger *et al.* 1994). Factors which influence lymph flow such as exercise, are thought to increase creatine kinase clearance (Cannon *et al.* 1990). Lymph flow induced changes in CK in rodents have been used as criticisms of this enzyme as a muscle damage marker (Komulainen & Vihko 1994). However the lymph clearance effect appears to differ between species, as several reports show performance of non traumatic exercise in active humans does not elevate CK (Ahlborg & Brohult 1967, Berg & Harlambie 1978, Viguie *et al.* 1993).

It has been proposed that the amount of damaged muscle (in grams), in a specific individual, can be calculated from the cumulative activity in serum/plasma, plasma volume and the activity of creatine kinase per gram muscle (Janssen *et al.* 1989). However such calculations tend to overestimate the extent of muscle damage by several orders of magnitude (Cannon *et al.* 1990, Komulainen & Vihko 1994, Volfinger *et al.* 1994). This may be due to the up-regulation of creatine kinase production in damaged fibres to combat membrane release. Thus elevated serum creatine kinase activity may be a manifestation of muscle damage, but is probably a poor index of the magnitude of tissue injury both within and between subjects. Therefore CK may be suitable non quantitative marker of muscle damage within, but not between subjects.

### 1.1.4 Measurement of β-Glucuronidase

β–Glucuronidase (βG) is an acid hydrolase (Wagner *et al.* 1978), which is present in lysosomes. These structures are found in normal muscle (Shannon *et al.* 1974), neutrophils and macrophages. Extracellular release of β-Glucuronidase can result from the activation of neutrophils and macrophages (Dean *et al.* 1979), or peroxidation of lysosomal

membranes (Fong *et al.* 1973). Thus, elevated serum βG activity may be indicative of increased lysosomal membrane permeability.

In rodents muscle βG activity has been reported to correlate significantly with the pathological state of the muscle following damaging exercise (Vihko & Salminen 1976). It has been proposed muscle βG activity provides a quantitative marker of muscle damage (Salminen & Kihlstrom 1985). This may be possible as the activation of lysosomes may reflect protein breakdown (Baracos *et al.* 1993) which may be related to the magnitude of damage. It has been suggested such biochemical quantification of muscle damage may overcome some problems associated with histological quantification techniques. Primarily these relate to the focal nature of infiltration within biopsy specimens and variability in the magnitude of cellular infiltration in serial sections (Faulkner *et al.* 1993).

### 1.1.5 Muscle Glucose-6-Phosphate dehydrogenase activity

Glucose-6-Phosphate dehydrogenase (G6PDH) catalyses the first step in the pentose phosphate shunt and has low activity in normal muscle; in contrast inflammatory cells demonstrate high activity (Fehr *et al.* 1989, Weiss 1989). Consequently elevation in G6PDH activity has been used as a marker of cellular infiltration in exercise damaged rodent muscle (Tullson & Armstrong 1981, Armstrong *et al.* 1983, Warren *et al.* 1992).

### 1.1.6 Measurement of malondialdehyde

Bendich (1991) proposed increased levels of peroxidation products may represent an overwhelming of normal metabolic control mechanisms, which may result in the oxidation of polyunsaturated fatty acids. This results in the formation of numerous lipid hydroperoxides and malondialdehyde (MDA), and such events may be associated with exercise induced muscle damage (Kanter *et al.* 1988, Maughan *et al.* 1989). Although MDA is not a specific marker of free radical damage to

muscle, it may provide evidence for peroxidation when measured in combination with indices of muscle damage.

Most assays measuring MDA involve a reaction with thiobarbituric acid (TBA), to form an adduct with maximum absorbance at 532nm. Compounds other than MDA also react with TBA to produce adducts with similar absorbent properties e.g. carbohydrates, pyrmidines (Wade & van Rij 1988), thereby reducing assay specificity (Conti *et al.* 1991). To combat these problems high pressure liquid chromatography (HPLC) is recommended to separate the MDA-TBA adduct from its contaminants (Hoving *et al.* 1992, Halliwell & Chirico 1993).

### 1.1.7 Muscle soreness

The occurrence of muscle soreness (MS) following eccentric exercise is commonly accepted as evidence for muscle damage; although several reports show it does not correlate well with other damage indices (Rodenburg *et al.* 1993). Typically soreness is first experienced in the first 24 hours following exercise, subsequently subsiding over the following 5 to 7 days (Byrnes & Clarkson 1986, Clarkson *et al.* 1992). The perception of soreness is thought to be produced by type III and IV muscle afferents, which can be sensitised by a variety of compounds including prostaglandins, arachidonic acid and potassium (Hayward *et al.* 1991). Any one, or all of these compounds could potentially accumulate in damaged muscle (Armstrong 1984) and contribute to sensations of pain (Edwards 1988).

It is clear soreness is maximal before the peak in histological or ultrastructural damage (Jones *et al.* 1986) and therefore is not directly related to these events. It is likely the MS experienced after damaging exercise is the summation of events associated with connective tissue damage (Stauber 1989) and arachidonic acid and prostaglandin synthesis (Hayward *et al.* 1991). It has also been suggested swelling may also contribute to muscle soreness (Stauber *et al.* 1990). More recent studies using magnetic resonance imaging have shown muscle soreness precedes oedema following high force eccentric actions using the elbow flexors (Rodenburg *et al.* 1994). These authors proposed that a factor other than oedema produced the initial rise in muscle soreness following high force

eccentric contractions. A number of factors may contribute to the overall perception of muscle soreness. It is likely the relative contribution of any one factor to total muscle soreness will differ over time and between individuals.

Muscle soreness is often measured using visual analogue scales (Rodenburg *et al.* 1994), where a number is chosen based on the perception of soreness. There are many limitations to such methods, but soreness may be a useful marker of tissue injury when used in conjunction with other damage indices

## 1.2.0 Study Aims

Several mechanical factors have been associated with muscle damage during eccentric muscle actions. Typically these have been studied using *in vitro* and *in situ* muscle preparations from small mammals. There are few well controlled studies investigating the mechanics of muscle damage in humans. The objectives of the first two studies were to manipulate strain and force in human muscle, independently of other mechanical factors, thereby allowing their effects on muscle damage indices to be investigated.

It is apparent a number metabolic events also contribute to exercise induced muscle injury. Experiments using rodents suggest free radicals may be involved in the degenerative changes following damaging exercise. Many of the free radical, muscle damage and antioxidant markers used in muscle damage studies have been criticised for their lack of specificity and validity (Kanter 1995). More importantly perhaps, most investigations in humans have assessed free radical and muscle damage indices in blood and urine rather than the damaged tissue. To overcome some of these problems more specific assays were used for the assessment of free radical, muscle damage and antioxidant status. When possible, assessment of muscle damage was determined simultaneously in both damaged tissue and blood.

# CHAPTER 2

## Materials and methods

## 2.1 Eccentric actions

Eccentric muscle actions were performed using a Kin-Com III isokinetic dynamometer (Chattecx Corp., Chattanooga, USA). This was controlled by a computer (Relisys, Milpitas, USA) running System Breeze Software (Chattecx Corp., Chattanooga, USA), which was also used for analysis of force data.

Subjects were firmly strapped to the dynamometer to minimise body movements and to isolate the knee extensor (KE) musculature. Typical positioning for exercise is shown in Fig. 2.1 . During exercise, each subject was verbally encouraged to produce maximum effort during the eccentric phase of each repetition.

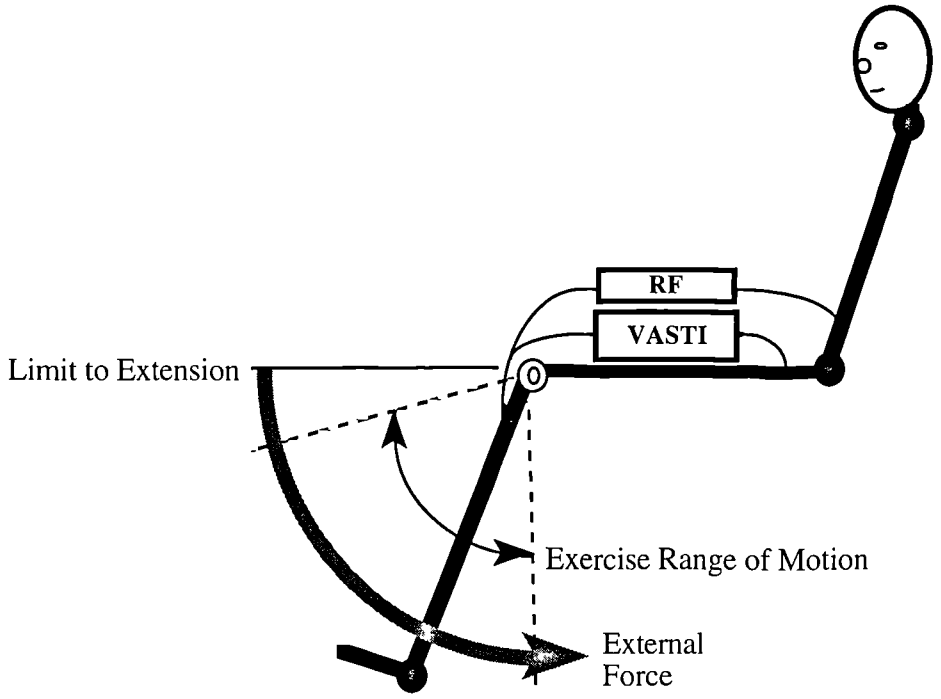


**Fig. 2.1 Schematic diagram showing the production of eccentric muscle actions using an isokinetic dynamometer**

Force is generated by the *rectus femoris* (RF) and *vasti*, which attempt to produce extension at the knee joint. A greater force is applied in the opposite direction by the isokinetic dynamometer, to produce flexion at the knee joint. Thus the knee extensors perform an eccentric muscle action.

## 2.2 Isometric force measurements

For measurement of isometric KE force subjects were positioned as shown in Fig. 2.1, using the strain gauge system.

When the strain gauge system was used to measure KE force this was calibrated daily with known weights. The strain gauge output was amplified (MacLab Bridge Amp), interfaced to a computer (MacLab/4 MkIII analogue digital converter with Macintosh computer) and analysed with Chart 3.3.3 software (AD Instruments, Hastings, East Sussex, UK). The strain gauge was used in preference to the Kin-Com for measurements of isometric force due to higher signal sampling frequency, which allowed additional contractile measures to be evaluated. The relationship between applied force and strain gauge output was linear within the physiological range of applied forces. A calibration curve is shown in Appendix 7.

Maximum voluntary contractile force was determined by verbally motivating subjects to maximally contract the KE for 3 seconds, whilst recording the maximum tetanic force. Following a one minute rest period this procedure was repeated to calculate the average force produced in two contractions.

## 2.3 Percutaneous electrical myostimualtion

Percutaneous electrical myostimulation (PES) was used to manipulate the force produced by the KE. Muscles were stimulated using a Bioscience 200 stimulator (Bioscience, Sheerness, Kent, UK) producing a unidirectional square wave signal with a pulse width of 0.5ms. Stimulation was applied *via* large copper electrodes, with minimum dimensions of 15 x 10cms (0.2mm thick). These were contained within water soaked felt pouches and affixed proximally and distally over the knee extensors with elasticated straps. Subjects were habituated to PES on at least two visits to the laboratory before exercise.

The methods employed were similar to those described by Saxton (1994) although several modifications were made. The 'dry connections' between the stimulator output, cabling and the electrodes were hardwired. The aluminium foil electrodes were replaced with the

copper electrodes described previously. These modifications allowed all subjects to be reliably stimulated in excess of 50% of MVC (at 100Hz), both isometrically and during exercise.

The higher force generated is thought to reduce the relative contribution of viscoelastic force to the variability in isometric force measurement (Bulow *et al.* 1993). A cable, pulley and weight system was constructed to 'pre stress' the leg strain gauge system. This was attached to the leg to exert a constant force of 20 Newtons, even with the leg relaxed. This system reduced both the variability in baseline force measurement with the leg relaxed and inertial overshoot during muscle contraction. A schematic diagram is shown in Fig. 2.3.

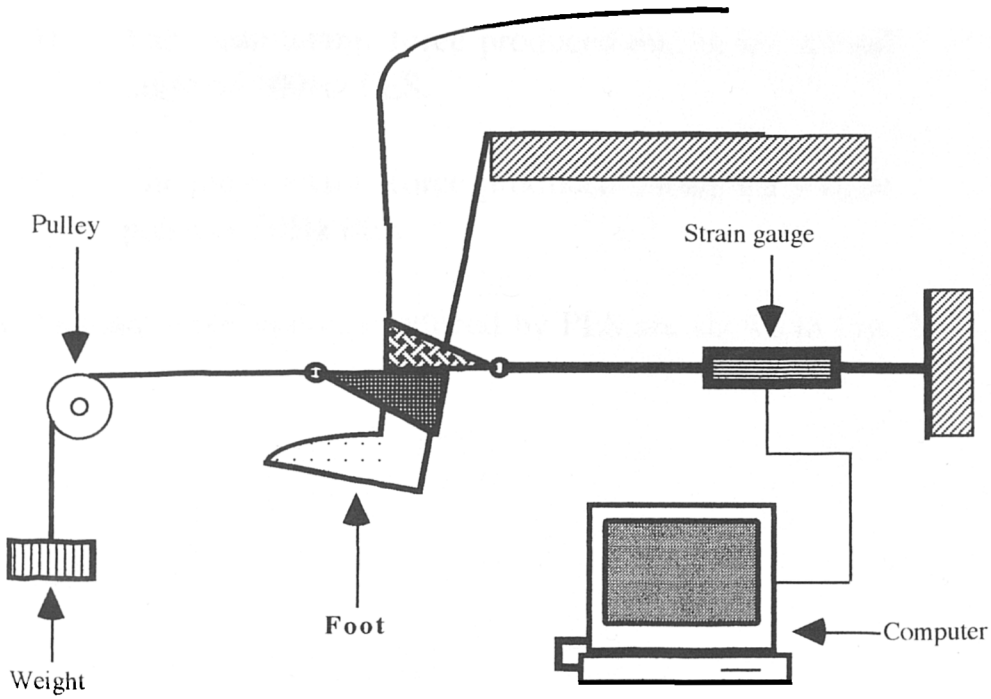


**Fig. 2.2 Schematic representation of the leg strain gauge system.**

Volunteers were familiarised to isometric strength testing and PES on at least two visits to the laboratory before exercise. The sessions involved each volunteer habituating themselves to PES by manipulating the voltage to produce approximately 50% of maximum voluntary force. After familiarisation the following contractile parameters were made in

duplicate, measures 3 and 4 were used to calculate the 20:100 force ratio, which was used as an index of LFF.

1) Maximum voluntary contractile force (MVC) was measured by motivating subjects to maximally contract the KE for 3 seconds, whilst recording the maximum tetanic force.

2) MVC with superimposed myostimulation was determined by applying a 1 second pulse of 100Hz stimulation during a 3 second MVC, at a voltage sufficient to induce at least 50% of the subjects MVC on that day (MVS).

3) The mean tetanic force produced during a 1 second pulse of 100Hz PES.

4) The mean tetanic force produced during a 1 second pulse of 20Hz PES.

Typical tetanic force curves produced by PES are shown in Fig. 2.3.

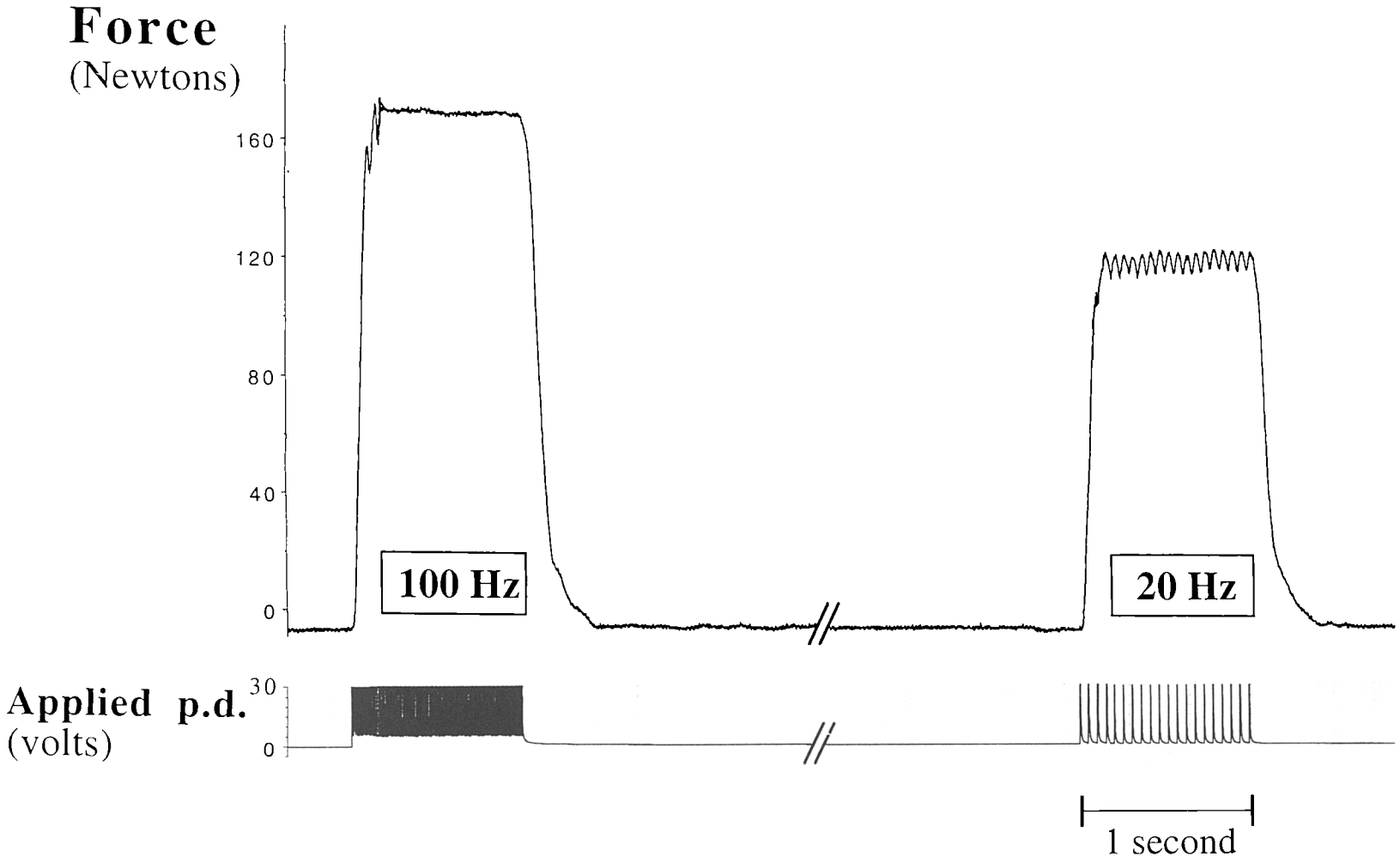


**Fig. 2.3 Isometric force trace (*top*) and stimulator output (*bottom*), during percutaneous electrical myostimulation of the knee extensors at 100Hz (*left*) and 20Hz (*right*).**

## 2.4 Muscle Soreness

Muscle soreness was determined using a questionnaire, shown in Appendix 1, which was similar to that used previously by Byrnes *et al.* (1985). Modifications were made to allow assessment of soreness at an increased number of sites on the (KE). Subjects were requested to palpate 8 muscle sites on the thigh in a seated position, with the knee flexed. A total of 6 sites were located above the belly and distal regions of the vasti, with 2 sites above the knee flexor muscles. Perceived muscle soreness was reported using a visual analogue scale between 1 (no soreness) and 10 (very very sore). Soreness measures were always collected prior to assessment of muscle function, when these data were collected on concurrent days.

## 2.5 Blood collection

Blood was drawn after cleansing the anticubital fossa with a swab (saturated with 70% isopropyl alcohol BP) and application of a torniquet above the distal region of the biceps. Blood was taken from a prominent forearm vein into a 10 ml syringe using a 0.8 mm (21 gauge needle). Three milliliters of each blood sample was dispensed into a 5ml di-potassium ethylenediaminetetraacetic acid (EDTA) tube (LIP 2298 KE/S, LIP Plastics, Shipley, UK), with the remaining blood dispensed into a 10 ml plain tube (LIP Z/10/T, LIP Plastics).

EDTA tubes were immediately centrifuged at 1,500*g* for 10 minutes (Megafuge 1.0, Heraeus Sepratech No.3360, Germany). Serum tubes were allowed to clot at room temperature prior to centrifugation, using the same protocol. Plasma and serum were removed using 3 ml disposable pipettes (No.28902, LIP Plastics) and transferred to 1.5 ml plastic vials. Plasma samples were stored at -80°C to minimise peroxidation during storage, while serum samples were stored either at -20°C or -80°C.

## 2.6 Creatine kinase activity

Creatine kinase (EC 2.7.3.2, ATP: Creatine Phosphotransferase) activity was determined *via* an enzyme linked reaction, using a diagnostic kit (Sigma No. 47-20, Sigma Chemicals, Poole, UK). The reaction sequence is described below.

$$\text{Creatine Phosphate + ADP} \xrightarrow{\textit{Creatine kinase}} \text{Creatine + ATP}$$

$$\text{ATP + Glucose} \xrightarrow{\textit{Hexokinase}} \text{ADP + Glucose-6-Phosphate}$$

$$\text{Glucose-6-Phosphate + NAD} \xrightarrow{\textit{G6PDH}} \text{6-PG + NADH}$$

Creatine kinase catalyses the reaction between creatine phosphate and adenosine diphosphate (ADP), forming creatine and adenosine triphosphate (ATP). The ATP formed is utilised to phosphorylate glucose, producing glucose-6-phosphate (G-6-P) in the presence of hexokinase (HK). Subsequently, G-6-P is oxidised to 6-phosphogluconate (6-PG) in the presence of nicotinamide adenine dinucleotide (NAD). This reaction is catalysed by glucose-6-phosphate dehydrogenase (G6PDH). During oxidation, an equimolar amount of NAD is reduced to NADH increasing the absorbance at 340 nm. The rate of change of absorbance is directly proportional to creatine kinase activity.

The reagent consisted of :-

Creatine phosphate 30mmol.l$^{-1}$, ADP 2mmol.l$^{-1}$, adenosine monophosphate 5mmol.l$^{-1}$, NAD 2mmol.l$^{-1}$, N-acetyl-Cysteine 20mmol.l$^{-1}$, HK (yeast) 3000 U.l$^{-1}$, G6PDH (L.m) 2000 U.l$^{-1}$, magnesium ions 10 mmol.l$^{-1}$, D-glucose 20 mmol.l$^{-1}$, Di (adenosine 5') pentaphosphate 10 µmol.l$^{-1}$, EDTA 2 mmol.l$^{-1}$, buffer pH 6.7$\pm$0.1, sodium azide 0.05%.

Serum samples (20 μl) were added to 1 ml of creatine kinase reagent (at 30°C) in a 5 ml test tube, sealed with laboratory film (Parafilm M, American National Can, Greenwich, USA) and mixed. The reactants were then incubated at 30°C for 3 mins in a water bath, before being transferred to a thermally controlled cuvette in a spectrophotometer (Beckman DU 70, Beckman Scientific Instruments, Fullerton, USA). Absorbance measurements were made every 10 secs at 340 nm for 2 minutes, while the temperature was held constant at 30°C. Using deionised water as a reference creatine kinase activity was calculated from the change in absorbance per min x 8200. Using a serum sample (with activity of 298 $IU.l^{-1}$), 6 repeated measurements resulted in an inter assay CV of 3.8%. At least duplicate analysis were made of each sample, and creatine kinase activities were calculated as a mean of two values that differed by no more than 10% of the lower value.

## 2.7 Glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase (G6PDH, D-glucose-6-phosphate: oxireductase, EC 1.1.1.49) activity was determined *via* the reduction of NADP to NADPH using a diagnostic kit (Sigma No. 345-UV, Sigma Chemicals). The reaction sequence is described below.

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{G6PDH} \text{6-PG} + \text{NADPH} + H^+$$

G6PDH oxidises glucose-6-phosphate (G-6-P) to 6-phosphogluconate (6-PG) and reduces NADP to NADPH.

The reagents consisted of :-

G-6-PDH reagent (NADP 1.5 $mmol.l^{-1}$ and maleimide 12 $mmol.l^{-1}$).

G-6-PDH substrate solution (Glucose-6-phosphate 1.05 $mmol.l^{-1}$, buffer, magnesium salt and sodium azide).

The sample 25 µl was added to 0.5 ml of G6PDH reagent and allowed to stand at room temperature for 10 mins, before addition of 1 ml of G6PDH substrate solution. The reagents were then mixed, incubated at 37°C for 5 mins, before being transferred to a thermally controlled cuvette in a spectrophotometer (Beckman DU 70). Absorbance measurements were made every 20 secs at 340 nm for 5 minutes, while the temperature was held constant at 37°C. Using deionised water as a reference G6PDH activity was calculated from the change in absorbance per min x 9807. Using a rat muscle homogenate (activity 55 pmol NADP reduced.$l^{-1}$), 6 repeated measurements resulted in an inter assay CV of 6.3%.

## 2.8 β-Glucuronidase activity

β-Glucuronidase (GRS, EC 3.2.1.31) activity (βG) was determined *via* the cleaving of phenolphthalein from phenolphthalein-mono-β-glucuronic acid (β-GA), using a diagnostic kit (Sigma No. 325). The reaction sequence is described below.

$$\text{Phenolphthalein-β-GA} \xrightarrow{\beta\text{-Glucuronidase}} \text{β-GA} + \text{Phenolphthalein}$$

Reagents consisted of :-

Acetate buffer solution (sodium acetate 0.2 mmol.$l^{-1}$, pH 4.5 @ 25°C and chloroform).

Phenolphthalein glucuronic acid solution (phenolphthalein-mono-β-glucuronic acid 0.03 mol.$l^{-1}$, pH 4.5 @ 25°C).

2-amino-2-methyl-1-propanol (AMP) buffer (AMP 0.1 mol.$l^{-1}$, pH 11 and sodium lauryl sulphate 0.2%).

The 'reagent blank' comprised 0.3 ml acetate buffer solution, 0.1 ml glucuronic acid solution and 0.1 ml of water. The 'serum blank' was comprised of 0.3 ml acetate buffer solution, 0.1 ml water and 0.1 ml of serum. The 'test' was comprised of 0.3 ml acetate buffer solution, 0.1

ml glucuronic acid solution and 0.1 ml of serum. Reagents were mixed by vortexing and heated at 56°C in sealed glass vials for 1 hour. Immediately following incubation 2.5 ml of AMP buffer was added to each tube and gently mixed to dissolve the milky precipitate. Absorbance was measured at 550 nm using deionised water as a reference. The relationship between the phenolphthalein concentration and absorbance was linear (r=0.999), and is shown in Appendix 2. Liberation of 1 μg of phenolphthalein per litre, per hour, at 56°C, was defined as one Sigma unit of activity. Using a serum sample with activity of 16,500 Sigma Units.$l^{-1}$, 6 repeated measurements resulted in an inter assay CV of 3.7 %.

## 2.9 Uric acid concentration

The serum concentration of uric acid was determined using a diagnostic kit (Sigma No. 686). This involves the oxidation of uric acid using uricase to form hydrogen peroxide. In the presence of peroxidase this catalyses a reaction between 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydrobenzene-sulfonate (DHBS) to form a quinoneimine dye with maximum absorbance at 520nm. The reaction sequence is described below.

$$\text{Uric Acid} + O_2 + H_2O \xrightarrow{\textit{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{DHBS} \xrightarrow{\textit{Peroxidase}} \text{Quinoneimine Dye} + H_2O$$

The reagents consisted of :-

Uric acid reagent A (4-Aminoantipyrine 0.375 mmol.$l^{-1}$, 3,5-Dichloro-2-hydroxybenzenesulfonate 2.5 mmol.$l^{-1}$, horseradish peroxidase 6250 U.$l^{-1}$ and buffer pH 7.2±0.1).

Uric acid reagent B (Uricase [c. utilis] 675 U.$l^{-1}$, Buffer pH 7.2±0.1 and sodium azide 0.05%).

Serum samples or blank (25 µl of deionised water) were added to reagent 'A' (0.8 ml) in labelled cuvettes and mixed. Absorbance was then measured at 520 nm (Beckman DU 70) using deionised water as a reference. This value was multiplied by 0.805 to compensate for the dilution which occurs with the addition of reagent 'B', giving the initial corrected absorbance IA. Reagent 'B' (2 ml) was then added to each cuvette, mixed and incubated for 10 mins at room temperature. Absorbance was then re-measured at 520 nm, giving the final absorbance (FA). The net change in absorbance was calculated by subtracting IA and the change in absorbance for the blank from FA. The relationship between the uric acid concentration and absorbance at 550 nm was linear (r=0.998), within the physiological range of uric acid concentrations (Appendix 3). Using a 296 µmol.l$^{-1}$ serum sample 6 repeated measurements resulted in an inter assay CV of 4%.

## 2.10 Total Antioxidant capacity (TAC)

Samples were analysed for total antioxidant capacity using the enhanced chemiluminescent method of Whitehead *et al.* (1992), utilising 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as an external standard.

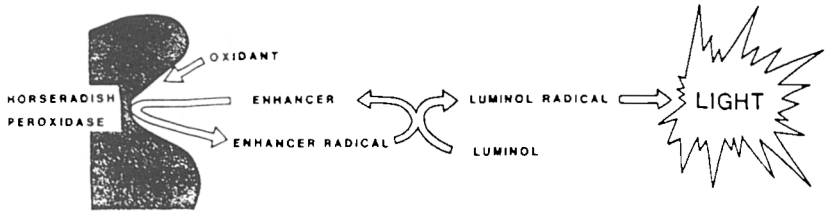


**Fig. 2.4 Schematic representation of the enhanced chemiluminescent reaction** Reprinted from Whitehead *et al.* (1992).

Light emission occurs when the chemiluminescent substrate (luminol) is oxidised by hydrogen peroxide or perborate, in a reaction catalysed by horseradish peroxidase (HRP). The stability and intensity of light output is enhanced by the addition of *p*-iodophenol. Continuous light output depends upon on the constant production of free radical intermediates derived from *p*-iodophenol, luminol and oxygen. For this reason light

emission is sensitive to chain breaking antioxidants (i.e. low molecular weight antioxidants) but will be restored when the added antioxidants have been consumed. As the generation of radical intermediates is constant the time period of light suppression is directly related to the amount of antioxidant present.

The reagents consisted of :-

Amerlite signal reagent buffer (comprised of luminol, *p*-iodophenol and oxidant), from Kodak Clinical Diagnostics, Amersham, UK.

HRP conjugate (comprised of 5 μl of mouse IgG HRP linked whole antibody from sheep dissolved in 20 ml of deionised water), from Amersham International, Amersham, UK.

80 μmol.l$^{-1}$ Trolox solution (comprised of 20 mg of Trolox in 1 litre of deionised water), from Aldrich Chemicals (Gillingham, Dorset, UK).

In a cuvette (No. 68.750, 51mm x 12mm diameter, Sarstedt, Germany) signal reagent buffer (100 μl) was added to 800 μl deionised water. Following addition of 100 μl of HRP reagent, the initial light output of the reactants was determined. This was performed using a BioOrbit 1250 luminometer (BioOrbit, Turku, Finland), employing a side window photomultiplier tube (EMI/Type 9781A, 94uA/lumen). The luminometer output was connected to a single pen chart recorder (Philips PM 8251, Philips, Holland), set at 5 volts with a paper speed of 20 mm.min$^{-1}$. The sample (20 μl) was added to the reactants and the light output was monitored until it returned to 10% of its' initial value. Original records showing light recovery following addition of Trolox, serum and aqueous and bound muscle extracts are shown in appendices 4b, 4c, 4d and 4e respectively.

The relationship between antioxidant capacity and the distance to 10% light output (i.e. time) was linear (r=0.999), and is shown in Appendix 4a. Antioxidant capacity was expressed as Trolox equivalents (Trolox Eq.), relative to sample volume or tissue mass, with each analysis performed in duplicate. Evaluation of the TAC of 20 samples of muscle aqueous extracts, bound extracts and serum revealed intra assay CVs of 0.4%, 1.4% and 0.9% respectively. Using a serum sample (with

antioxidant capacity of 428 μmol.Trolox Eq.$l^{-1}$), 6 repeated measurements resulted in an inter assay CV of 4%.

*Development work for assessment of tissue TAC*

The application of enhanced chemiluminescent techniques to measure tissue TAC have not been reported previously, therefore development work was required to assess aqueous and bound TAC. This was performed using rodent *soleus* muscle prior to analysis of human tissue. A glass on glass homogeniser was used as this was found to be more effective in breaking down muscle connective tissue components than Teflon pestle homogenisers.

Human biopsy specimens weighed between 12.8mg and 73.5mg, therefore it was important to establish that the preparation procedures allowed all samples to be within the linear range of the assays. This was performed by preparing and analysing rodent tissue weighing 12mg and 100mg, which exceeded the weight range of human biopsies). From the data obtained using 100mg of rat tissue it was estimated that all samples were would be within the linear range of the assays even if a three fold elevation in activity, concentration or quenching occurred. Increases above this level would necessitate sample dilution for aqueous TAC, however if this arose enough 'B' sample would still remain to allow performance of duplicate analyses. To measure bound antioxidants the muscle pellet weight must be accurately determined. After decanting the aqueous supernatant layer, aqueous antioxidants are still present in the pellet. It is essential to remove these compounds to prevent 'contamination' of antioxidants in the bound phase. This was performed by re-suspending the pellet in 1 ml of buffer, mixing, centrifugation and decanting the upper layer. When this procedure was performed twice it was estimated the concentration of aqueous antioxidants in the muscle pellet was reduced approximately 400 fold, thereby preventing interference when measuring bound TAC. The pellet was freeze dried at -20°C for 5 hours (in a pre-weighed vial) to allow the dry mass of tissue to be accurately determined. To verify that pellets were desiccated within this time period pellets were weighed after 5 hours and 7 hours of freeze drying. This revealed no further weight loss after 5 hours, suggesting there was no additional water loss in this period. DMSO was selected to extract the pellet antioxidants as this solubilises lipid bound compounds, thereby allowing the radical scavenging capacity of vitamin

E and β-carotene to be determined in aqueous conditions. The effectiveness of the extraction process could not be evaluated however as the membrane content of antioxidants was not known.

## 2.11 Muscle sulphydryl concentration

The muscle concentration of total mercaptans were determined using a colourimetric kit (GSH-400, Bioxytech, Bonneuil, France). The assay is based on the formation of substitution products (thioethers) between 4-chloro-1-methyl-7-trifluromethyl-quinolinium (reagent R1) and all mercaptans (sulphydryls) present in the sample.

$$R1 + \text{sample} \Rightarrow \text{thioethers}$$

To precipitate proteins 180 μl of ice cold 8.3% Metaphosphoric acid was added to 150 μl of ice cold muscle homogenate in a vial. This was then sealed, vortexed for 10 seconds and centrifuged at 4°C at 13,000*g* for 10 minutes. 300 μl of the aqueous upper layer was removed and immediately added to 600 μl of buffer and 50 μl of reagent R1. After mixing and incubation in the dark at 25°C, for 15 minutes, absorbance was measured at 356 nm using a Cecil C1010 spectrophotomer (Cecil Instruments, Cambridge, UK).

Within the physiological range of muscle sulphydryl concentrations (assayed at 356 nm) there was a linear relationship ($r=0.999$) between concentration and absorbance (Appendix 5). Using a 25 $\mu mol.l^{-1}$ standard, 7 repeated measurements resulted in an inter assay CV of 1%.

## 2.12 Malondialdehyde concentration

The concentration of malondialdehyde (MDA) was measured using high pressure liquid chromatography (HPLC) with fluorescence detection, using a modification of the method described by Young & Trimble (1991). Pre column derivitisation involves the formation of an adduct with fluorescent properties, which is outlined overleaf.

MDA + TBA ⇨ MDA-TBA adduct

Reagents consisted of :-

Thiobarbituric acid (TBA) solution (40 mmol.l$^{-1}$)

Phosphoric acid solution (1.22 mol.l$^{-1}$)

Tetramethoxypropane standards

All solvents were of HPLC grade and obtained from BDH chemicals (Poole, Dorset, UK), reagents were obtained from Sigma Chemicals. The initial reaction involved mixing 250µl of phosphoric acid solution with 450µl of HPLC grade water, 50µl of sample/standard and 250µl of 0.40mmol.l$^{-1}$ TBA*. The reactants were heated at 100°C (Stuart Scientific SHT2D test tube heater, Camlab, Cambridge, UK) for 60 mins in sealed glass vials. After cooling to 4°C, 200µl of the reactants were added to 360µl of HPLC grade methanol, and 40µl of 0.5 mol.l$^{-1}$ NaOH.

To separate contaminating compounds with similar fluorescent properties, a Perkin Elmer HPLC system was used (Perkin Elmer, Connecticut, USA). This consisted of an ISS 200 Advanced LC sample processor, Model 250 Binary LC pump, LS 40 Fluorescence detector, PE Nelson 1022 computer, and integration software. The injection volume was 80µl for muscle samples and 50µl for plasma. The detector was set at excitation 532 nm and emission 553 nm. The mobile phase consisted of 50% phosphate buffer (25 mmol.l$^{-1}$, pH 6.5) and 50% HPLC grade methanol, with a flow rate of 0.8 ml.min$^{-1}$. Chromatographic separation was performed using a 25 cm x 4.6 mm (I.D.) C18, ODS2 analytical column, with 3 cm guard cartridge (Phase Separations, Flintshire, Deeside, UK).

A typical chromatogram for EDTA plasma is shown in Appendix 6a. The MDA concentration of MDA was determined using 1,1,3,3 Tetramethoxypropane as a standard. This combines stoichiometrically with TBA to form an adduct with properties indistinguishable from the MDA-TBA adduct using this assay. Contamination of reagents with TBA reactive material has previously been reported to interfere with MDA measurement (Gutteridge & Tickner 1978). When substituting the 'sample' with water there was no detectable fluorescence, suggesting the reagents were free of TBA reactive material. Co-elution of a plasma sample 'spiked' with standard, was used to identify the MDA-TBA peak

on the chromatogram, which was eluted at 4.7 minutes. Using the protocol described the reduced retention time is probably attributable to the use of a 25 cm column; which is 5 cms shorter than that described in the original method of Young & Trimble (1991).

Peak area was found to be more accurate than peak height in calculating the MDA concentration, this phenomenon may be due to column degradation during use. Fluorescence was proportional to the 1,1,3,3 Tetramethoxypropane concentration (r=0.999), within the physiological range of MDA concentrations (Appendix 6b). Evaluation of the MDA concentration of 20 different plasma samples revealed an intra assay CV of 2.1%. Using a plasma sample with an MDA concentration of 1.2 $\mu mol.l^{-1}$, 6 repeated measurements resulted in an inter assay CV of 8.6%.

* Following correspondence with (Young 1995) the 0.44 $mol.l^{-1}$ TBA solution originally described (Young & Trimble 1991) was replaced with a 40 $mmol.l^{-1}$ TBA solution. This allows the TBA to be easily dissolved, but gives comparable results to those reported for the original method (Young 1995).

## 2.13 Statistical analysis

Soreness data were collected using an ordinal scale of measurement and analysis with parametric statistics was considered inappropriate (Cohen & Holliday 1984). Therefore soreness data were analysed using Wilcoxon matched pairs tests.

Biochemical and functional data were collected using an interval ratio level of measurement, from at least 6 subjects and were considered suitable for analysis using parametric statistics. Data collected over more than two time points were analysed using repeated measures analysis of variance (ANOVA). Within group comparisons were performed using a one way ANOVA. Between group comparisons were made using a 2 way ANOVA. Where significance was attained within groups Newman-Keuls post-hoc tests were performed to compare specific time points. Where significant differences were attained using ANOVA between treatments, *t*-tests were used to compare specific time points. The accepted level of significance was $P<0.05$, above this $P$ values were considered non-significant (NS).

ANOVAs were performed using Super Anova software (Abacus concepts Inc., Berkeley, USA), Newman-Keuls tests were performed manually. All other statistical analyses were performed using Statview software (Abacus concepts Inc.).

# CHAPTER 3

## Comparison of eccentric knee extensor muscle actions at two muscle lengths on indices of damage and angle specific force production in humans.

Aspects of this study were presented at the XXIV European Muscle Conference, Florence, Italy (1995).

This study has been published in abstract form (R.B. Child, A.E. Donnelly, J.M. Saxton 1996 Comparison of eccentric knee extensor muscle actions at two muscle lengths on indices of damage and angle specific force production in humans. Journal of Cell Motility and Muscle Research 17: 148).

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## Summary

The effects of knee extensor (KE) length during eccentric exercise on indices of muscle damage and adaptation were investigated. Subjects (n=7) performed two bouts of 75 maximal voluntary eccentric muscle actions, at a knee joint angular velocity of 1.57 rad.sec$^{-1}$. One bout was performed with a KE at short muscle length (bout S), with a knee joint range of motion 2.79 to 1.40 radians (160$^{o}$ to 80$^{o}$); and a second with the contralateral KE at long muscle length (bout L), with a range of motion 2.01 to 0.7 radians (120$^{o}$ to 40$^{o}$). Maximum voluntary contractile force (MVC) was measured before and 5 minutes post exercise, and again on days 3, 5, 7, 10 and 12, at knee angles of 160$^{o}$, 120$^{o}$ and 80$^{o}$. KE muscle soreness (MS) was measured before exercise and on each day afterwards. Serum creatine kinase activity (CK) was measured before exercise and on days 3, 5, 7, 10 and 12.

MVC declined following each bout ($P$<0.01, repeated measures analysis of variance (ANOVA)), with a greater decline after bout L ($P$<0.05, ANOVA). MS was higher relative to bout S on days 1, 2, 3, 5 and 6 ($P$<0.05, Wilcoxon test). Although elevated after each exercise bout ($P$<0.01, ANOVA), CK did not differ between bouts. Functional muscle damage markers and MS suggest greater damage following bout L. Post-exercise angle specific force decrements suggest a transient increase in muscle length after bout L but not bout S.

## Introduction

Eccentric actions involve generating force while lengthening active muscle. Movements requiring rapid deceleration of body segments such as running and throwing have an eccentric component. Such activities can result in disruption to connective tissue (Takala *et al.* 1986) and diverse myocellular components including the sarcolemma, myofibrils and cytoskeleton (Hikida *et al.* 1983, Friden 1984, Stauber *et al.* 1990). Damaging eccentric exercise typically results in reduced maximum voluntary contractile force (MVC), delayed elevations in serum creatine kinase activity (CK) and muscle soreness.

The mechanisms by which eccentric actions result in muscle damage are unclear, although initial injury does appear to be mechanical in origin (McCully & Faulkner 1986, Armstrong *et al.* 1991, Friden & Lieber 1992). One possibility is that sheer forces are produced by tension imbalances between adjacent sarcomeres (Morgan 1990), which might cause muscle microtrauma when high muscle tension is produced (Armstrong *et al.* 1991, Friden & Lieber 1992).

Muscle held in short or lengthened positions rapidly changes its resting length by sarcomere addition or deletion, thereby optimising force production (Williams & Goldspink 1978). Muscle can also adapt to eccentric exercise, so that all damage indices are attenuated when subsequent eccentric muscle actions are performed (Ebbeling & Clarkson 1990, Clarkson *et al.* 1992, Brown *et al.* 1996). Friden (1984) provided histological evidence for series sarcomerogenesis in humans following eccentric training. Such an adaptation would increase cross bridge formation at long muscle lengths, which may protect sarcomeres from overstretching (Friden 1984) or sheer forces (Friden & Lieber 1992). These structural changes could be expected to persist for several weeks after the initial exercise bout and provide resistance to microtrauma during this period (Friden 1984). Based on histological observations in rats following repeated downhill running Lynn & Morgan (1994) stated "the training effect is an increase in the number of sarcomeres connected in series in a muscle fibre thus avoiding the descending limb" (of the length tension relationship). The relationship between force and sarcomere length is described in Fig. 1.4, which demonstrates the decline in isometric force in mammalian muscle when the saromere length exceeds 2.5μm.

Determining muscle or sarcomere length in humans presents several practical problems. Accurate estimates of sarcomere length *in vivo* cannot be performed on muscle collected using needle biopsy techniques (Mathieu-Costello *et al.* 1988). Functional manifestations of series sarcomerogenesis using high velocity dynamic contractions may be confounded by submaximal neuromuscular activation (Hortobagyi & Katch 1990, Narici *et al.* 1991) and preferential damage to type II fibres following eccentric exercise (Friden 1984). The *in vitro* relationship between isometric tension and sarcomere length for amphibian muscle was originally described by Gordon *et al.* (1966). In humans measurement of isometric MVC at different points on the length tension curve could be used to detect ML changes. This functional approach has previously been employed in humans (Herzog & ter Keurs 1988, Saxton & Donnelly 1996) and is considered sensitive enough to detect physiological changes in ML *in vivo* (Delp *et al.* 1990).

A model of muscle microinjury based on tension imbalances between sarcomeres is appealing, as such models predict an increase in damage when eccentric actions are performed at long length, as observed by Jones *et al.* (1989). In combination with ML increases the tension imbalance model has also been used to explain the decrease in muscle damage following training in rodents (Lynn & Morgan 1994). To date however, no study has investigated the effects of muscle length during eccentric exercise on the muscle length tension relationship in humans. It is also not known if the length dependent damage effect observed by Jones *et al.* (1989) using the arm biceps is seen in more trained locomotory muscles. The objectives of this study were to determine if (1) quadriceps damage was dependent upon its' length during eccentric actions and (2) using isometric force measurements on the length tension curve, determine if there was evidence for a sustained increase in muscle length as an adaptive response to eccentric exercise.

## Methods

*Subjects*

Untrained but physically active volunteers (4 male and 3 female), aged 24±2 years (mean ± SEM) each completed two bouts of eccentric exercise with the knee extensors (KE) at 'long' and 'short' muscle length. Subjects gave written informed consent to participate in the study which was authorised by Wolverhampton University Ethics Committee.

*Isometric force measurement (MVC)*

Familiarisation to isometric strength testing was performed on at least two visits to the laboratory before exercise, during which volunteers were firmly attached to the isokinetic dynamometer (described in section 2.1. MVC was measured by verbally motivating subjects to maximally contract the KE for 3 seconds, whilst recording the maximum tetanic force. During the first familiarisation session knee angle was measured using a goniometer aligned with the *greater trochanter*, *lateral condyle* and *lateral malleolus*. The goniometer was firmly affixed to the leg with tape while subjects maintained the anatomical standing position. Seating position was altered to suit each subjects anthropometric features and align the knee joint axis with that of the dynamometer lever arm. When an appropriate position was achieved, this was noted and used for all subsequent exercise and isometric testing measurements as required. Knee angle was also determined using the goniometer in both seated and prone exercise positions. These measurements were recorded with the lever arm exactly vertical, verified with the use of a spirit level to provide a reliable external source of calibration. In subsequent exercise and testing sessions the exact subject and dynamometer lever arm positions were maintained, thereby replicating the exact conditions for MVC determination.

In describing knee angle the convention was employed such that full extension was equivalent to 3.14 radians (180°). MVC

measurements were made on each leg at 2.79 radians when seated and 2.01 radians when prone. These measurements were used to establish the minimum dynamometer activation force or preload (as described by Narici *et al.* 1991) before each exercise bout.

When seated MVC measurements were made before and 5 minutes post exercise, and then again on days 3, 5, 7, 10 and 12. These time points were chosen to allow sufficient time for muscle recuperation between testing sessions, while at the same time giving sufficient data to allow force recovery to be monitored. Half a minute rest was allowed between each force measurement at knee angles of 2.79, 2.01 and 1.40 radians (160°, 120° and 80°). Mean MVC was calculated at these angles, at each time point, to provide an index of the overall force decrement.

*Exercise*

A one group two treatment crossover design was employed, randomised for order effects. Exercise bouts were performed two weeks apart, using the contralateral leg in the second bout.

In each bout subjects performed 75 maximal voluntary eccentric actions on a isokinetic dynamometer (using protocols similar to those described in section 2.1). The isometric preload was set so that 25% of each subjects' pre-exercise MVC at the start angle was attained before movement of the dynamometer lever arm. Each eccentric action was separated by an 8 second rest period. KE joint angular velocity was 1.57 $rad.sec^{-1}$ ($90^{o}.sec^{-1}$) for the eccentric action with the leg returned passively by the experimenter at a velocity of 1.05 $rad.sec^{-1}$ ($60^{o}.sec^{-1}$). Knee joint range of motion was 2.79 to 1.40 radians (160° to 80°) for exercise at 'short' muscle length (bout S) and 2.01 to 0.70 radians (120° to 40°) for exercise at 'long' muscle length (bout L).

Previous studies in our laboratory and in the literature (e.g. Westing *et al.* 1991) have shown peak KE force is produced between the knee angles of 90° and 110° during maximal voluntary eccentric muscle actions. To separate the effects of peak force from differences in muscle length during exercise there was an overlap in knee angle between 80° and 120°. This covered the angular range in which peak force is produced, thereby controlling for the effect of peak force during exercise.

*Serum creatine kinase activity (CK)*

Blood samples were drawn as described in section 2.5, before exercise, then again on days 3, 5, 7, 10, and 12 after exercise. Serum samples were analysed for creatine kinase activity as described in section 2.6.

*Muscle soreness (MS)*

Soreness was assessed before exercise and on each subsequent day at 8 muscle regions (6 extensor and 2 flexor), depicted on a questionnaire (Appendix 1). Soreness was determined using protocols described in section 2.4 . Average knee extensor muscle soreness was calculated on each day.

*Statistics*

Statistical analysis were performed as described in section 2.13, with all data presented as means±SEM.

## Results

*Pre exercise force production*

Between bout comparisons showed no differences in pre exercise MVC at knee angles of 160°, 120°, and 80°, however force differed between angles ($P<0.0001$, ANOVA). When averaged for both legs, force production at 160° was less than half that produced at 120° ($P<0.01$, Newman-Keuls test) and 80° ($P<0.01$, Newman-Keuls test). The difference in force production between 120° and 80° was not significant (Newman-Keuls test) (Fig. 3.1).

*Serum creatine kinase activity (CK)*

The individual changes in CK activity were highly variable with maximum values after exercise ranging from 80 to 8568 $IU.l^{-1}$. Therefore the results have been expressed relative to the maximal activity observed in each subject (Fig. 3.2). Before exercise CK was not significantly different between bouts (*t*-test), following exercise CK was elevated above baseline ($P<0.01$, ANOVA), although there was no difference in CK elevation between bouts (ANOVA). On day 5 highest CK was recorded at $995\pm628$ $IU.l^{-1}$ and $1965\pm1363$ $IU.l^{-1}$ after bouts S and L respectively.

*Muscle soreness*

KE muscle soreness (Fig. 3.3) was elevated above baseline on days 1 to 3 after bout S ($P<0.05$, Wilcoxon test) and days 1 to 6 after bout L ($P<0.05$, Wilcoxon test). Soreness was higher after bout L relative to bout S on days 1, 2, 3, 5 and 6 ($P<0.05$, Wilcoxon test). No soreness was recorded in the knee flexor muscles throughout the study.

*Post exercise force production*

The time course for MVC recovery is shown in Figs 3.4 and 3.5 . MVC (averaged for all joint angles) was reduced after each bout ($P<0.001$, ANOVA). The decline in MVC was greater after bout L than bout S ($P<0.05$, ANOVA) with significant differences post exercise and on day 3 (both $P<0.01$, $t$-test) and on day 5 ($P<0.05$, $t$-test).

After bout L mean MVC was below baseline post exercise, and on day 3 (both $P<0.01$, Newman-Keuls test). Post exercise MVC differed between knee angles ($P<0.01$, ANOVA), with a greater relative fall in MVC at 160° (50%) than observed at 120° (35%, $P<0.05$, Newman-Keuls test) and 80° (32%, $P<0.01$, Newman-Keuls test).

Following bout S mean MVC was below baseline post exercise ($P<0.05$, Newman-Keuls test), thereafter mean force loss was not different relative to baseline. There were no significant interactions between MVC an knee angle at any time point after bout S.

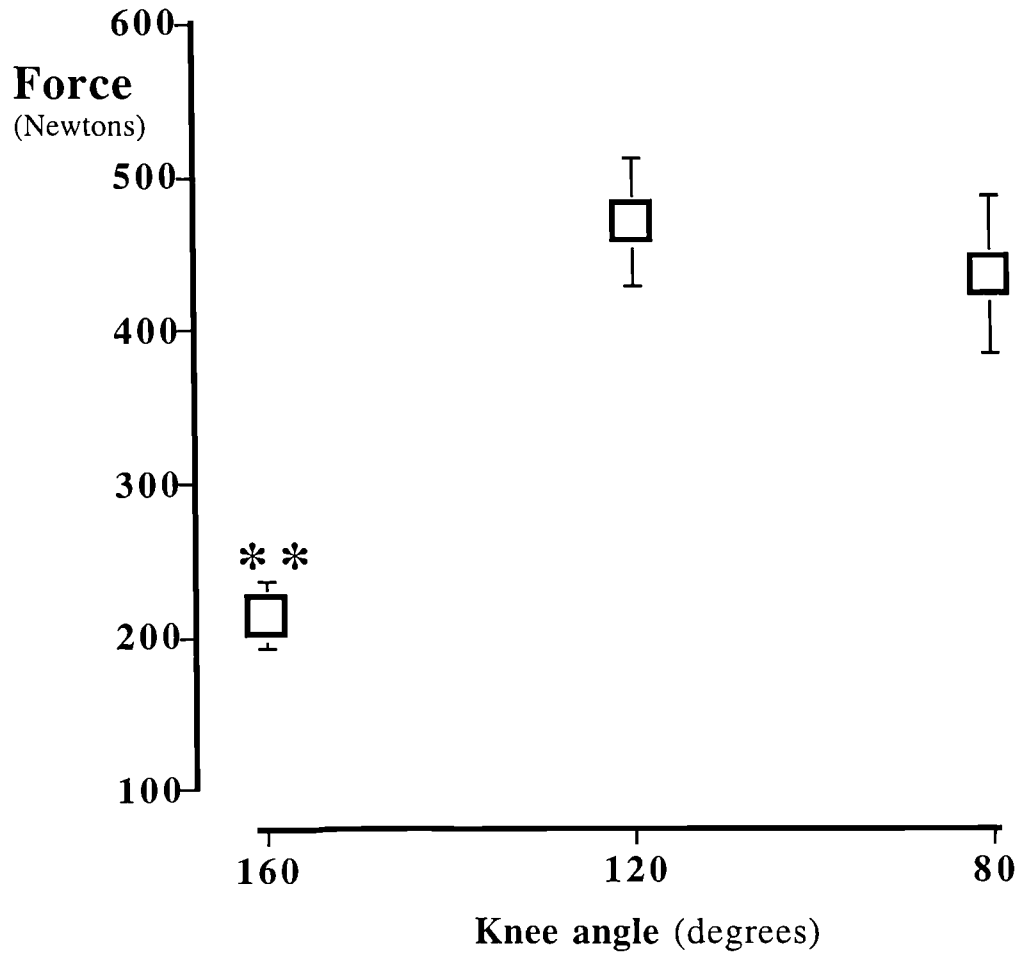


**Fig. 3.1 Pre exercise isometric force production at knee angles of 160°, 120° and 80°.**

*P*<0.0001, ANOVA between angles, ** lower force than at 120° or 80° (*P*<0.01, Newman-Keuls test).

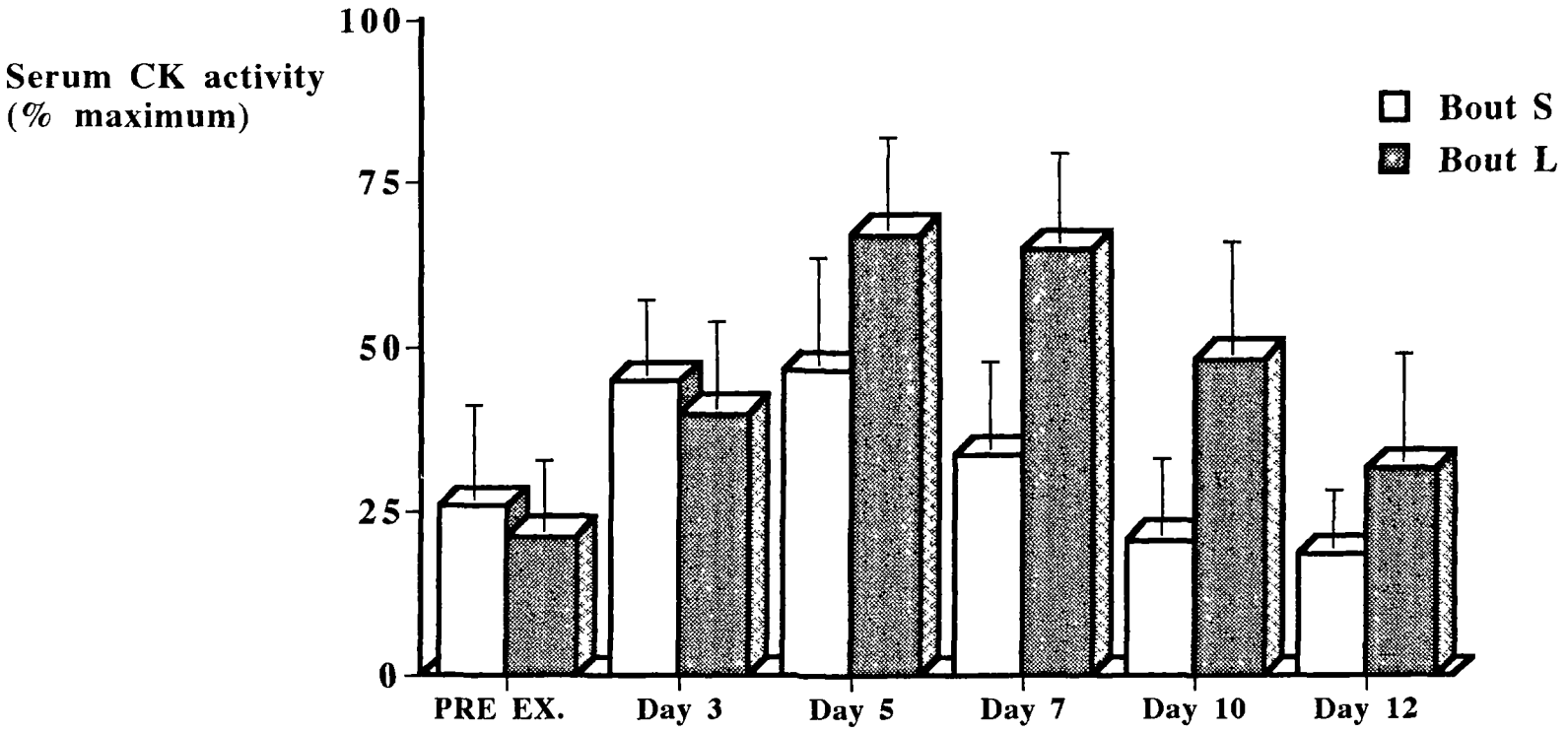


**Fig. 3.2 Changes in serum creatine kinase activity following eccentric exercise bouts at long (Bout L) and short (Bout S) knee extensor muscle lengths.**

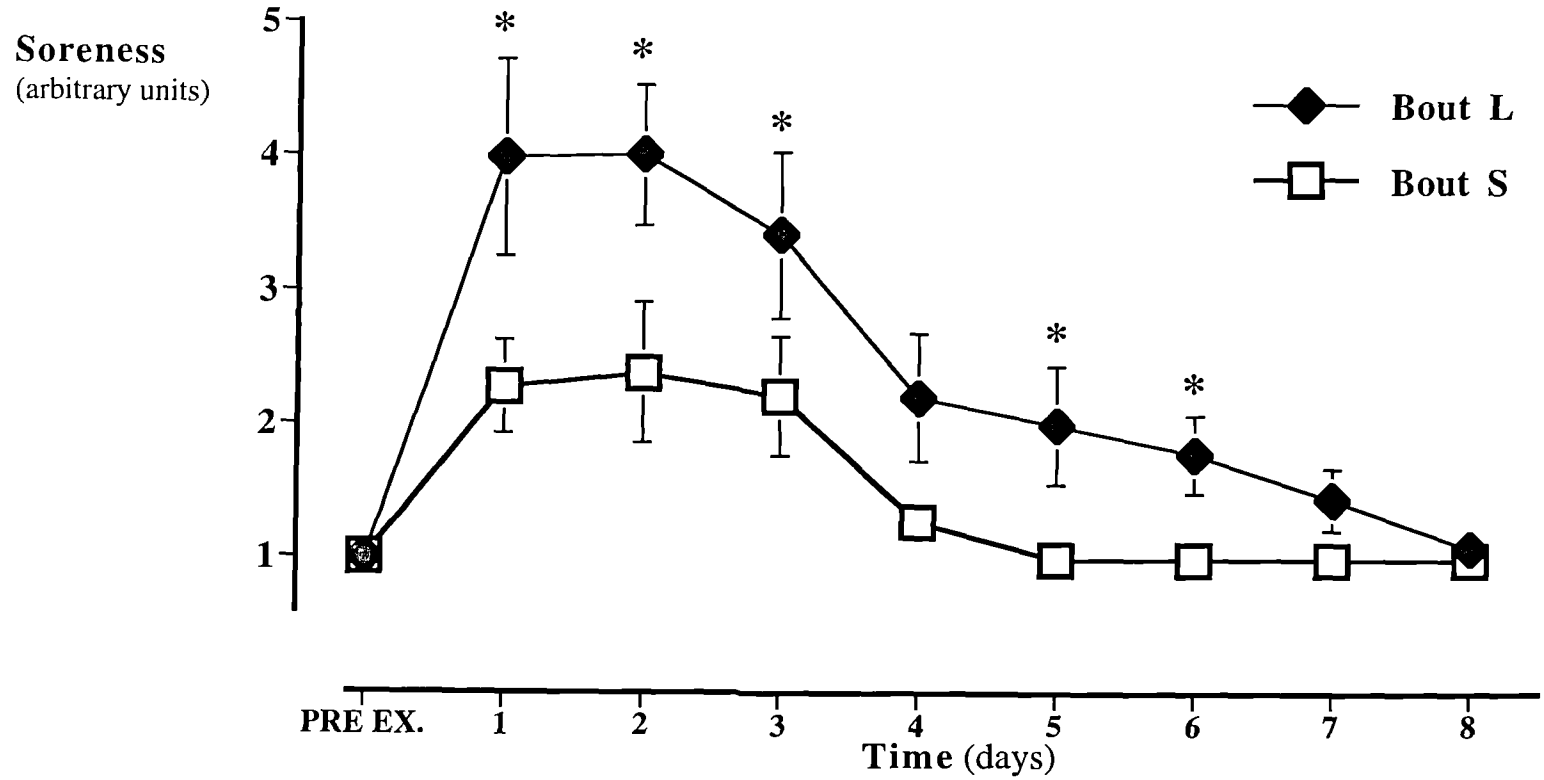


**Fig. 3.3 Changes in muscle soreness following eccentric exercise bouts at long (Bout L) and short (Bout S) knee extensor muscle lengths.**

* $P<0.05$ between bouts, Wilcoxon test.

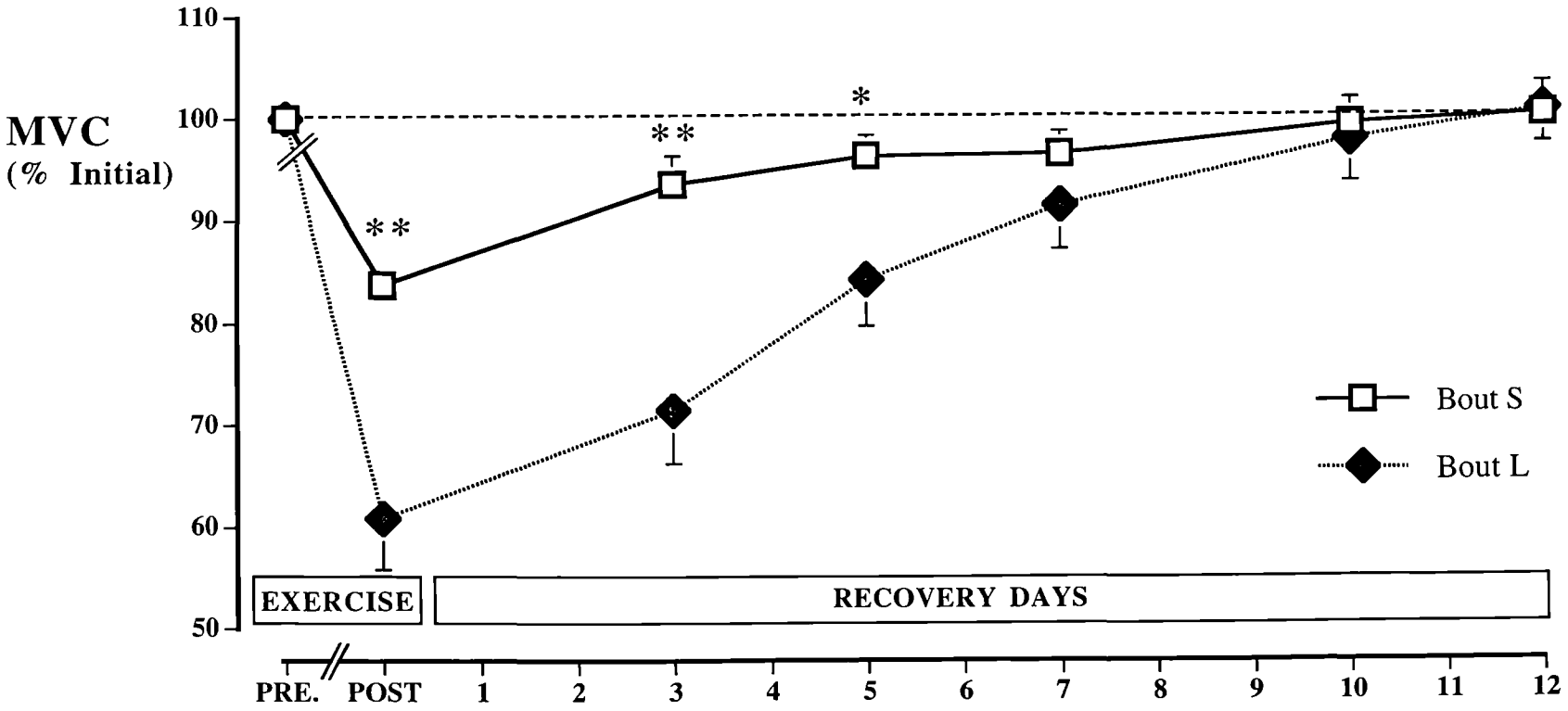


**Fig. 3.4 Mean force loss at all joint angles as a percentage of initial MVC.**

$P<0.05$ REANOVA between bouts with $^{*}P<0.05$, $^{**}P<0.01$ ($t$-test) at time points indicated.

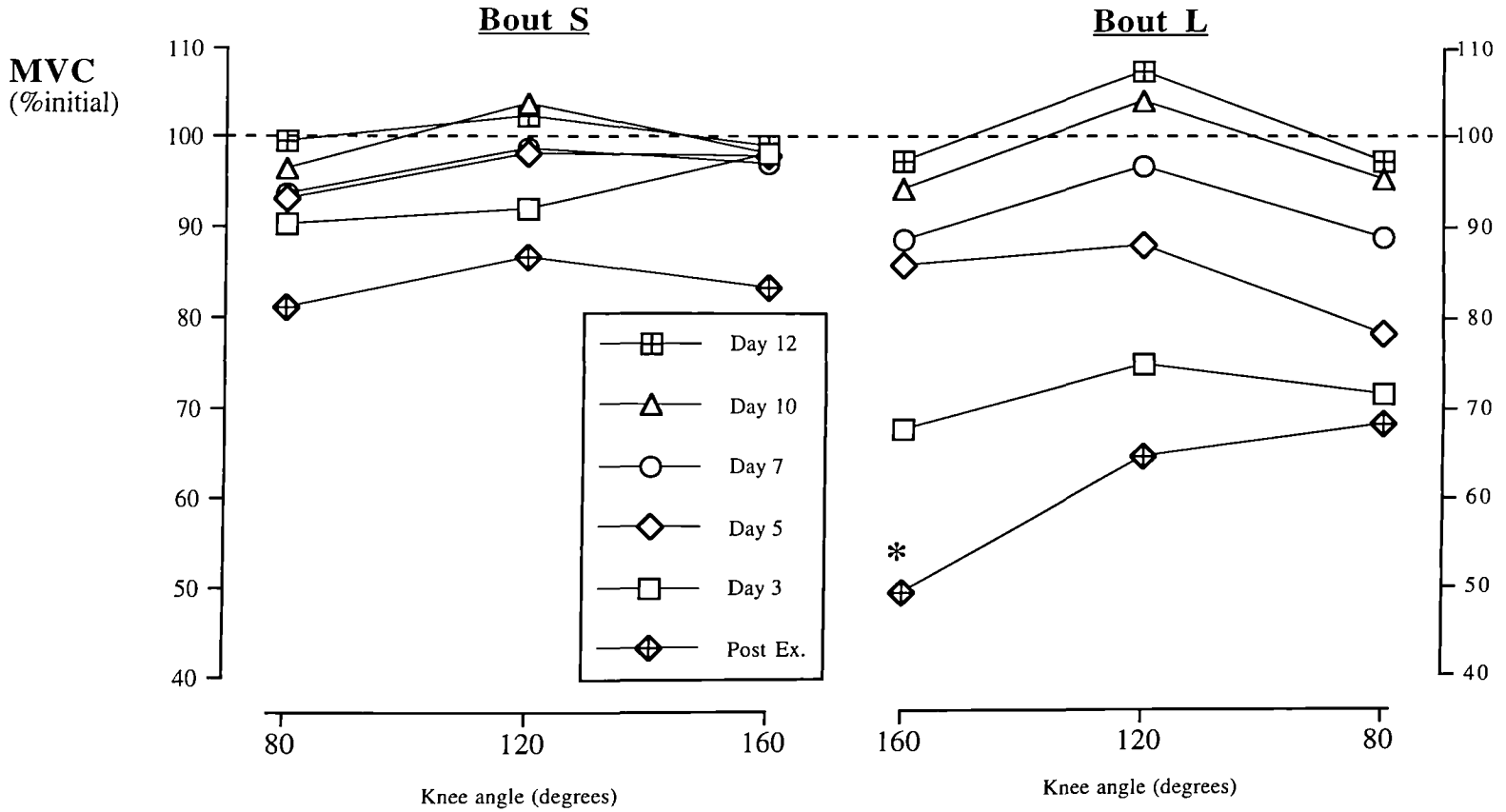


**Fig. 3.5 Force recovery at knee angles of 80°, 120° and 160°, following eccentric exercise bouts at long (Bout L) and short (Bout S) knee extensor muscle lengths.**

* Lower post exercise force than at 120° ($P<0.05$, Newman Keuls test) or 80° ($P<0.01$, Newman-Keuls test). Error bars have been omitted for clarity.

## Discussion

To date only the force length relations of the human *rectus femoris* have been determined *in vivo* (Herzog & ter Keurs 1988). For other human skeletal muscles force length predictions have been derived using modeling techniques based on cross bridge theory, or data from *in vitro* animal experiments (e.g. Gordon *et al.* 1966). The main determinant of active force production in poorly pennated muscle is sarcomere length (Willems and Huijing 1994), although aponeuroses also contribute to force production *in vivo*. Despite similarities between the muscle force joint angle relationship (Fig. 3.1) and classic length tension curves from isolated muscle, changes in isometric forces can only be used qualitatively to predict ML changes. Thus isometric force production at knee angles of 160°, 120° and 80° can only be considered in terms of 'short', 'medium' and 'long' KE lengths. However changes in force at these knee angles can be used qualitatively to determine small changes in muscle length (Delp *et al.* 1990).

This study was the first to assess the effects of muscle length during eccentric exercise, on angle specific force production in the following days. Both bouts L and S resulted in classic responses in all muscle damage indices, with a post exercise decline in MVC followed by a slow recovery and a delayed rise in serum CK and muscle soreness. All damage markers employed suggest that exercise at long muscle length produced greater damage to the quadriceps. This finding is consistent with previous observations using the arm biceps (Jones *et al.* 1989), but has not been reported previously for the KE muscles. Although the mechanism of damage is poorly understood there appears to be an additive effect of high muscle force and 'long' muscle length which increases muscle injury during eccentric contractions (Jones *et al.* 1989).

In some sarcomeres it has been proposed actin and myosin are pulled so far apart few, or no, cross-bridge interaction is possible (Jones *et al.* 1989, Morgan 1990, Lynn & Morgan 1994), which is described in Fig. 1.4. Morgan (1990) used the term "popping" to describe overstretched sarcomeres which failed to relocate on muscle shortening. Such sarcomeres might disrupt the adjacent sarcolemma or sarcoplasmic reticulum as a secondary event (Armstrong *et al.* 1991; Friden & Lieber

1992). Following eccentric exercise in humans, hyperextended sarcomeres have been observed in which cross bridges could not be formed (Newham *et al.* 1983b), while Hikida *et al.* (1983) have reported rupture of the sarcolemma. Such observations may give credence to the 'sarcomere popping theory' of muscle damage.

Immediately following bout S force loss was uniform at all joint angles. In contrast after bout L there was a greater relative force loss at the 160° knee angle. The greater functional impairment at the shortest muscle length might be attributable to an increase in ML, possibly resulting from damage to series structures (e.g. over-stretching of sarcomeres or damage to connective tissue). Stauber *et al.* (1990) have previously reported decreased muscle length following eccentric exercise, however this statement was based on a reduction in resting elbow angle, not on the muscles functional capacity. This conflict can be resolved, as in damaged muscle resting length could decrease due to calcium induced contracture (Brody 1969) while the functional length of the muscle is in fact increased.

There are several reviews documenting the prophylactic effect of a single bout of eccentric exercise on subsequent eccentric exercise induced muscle damage (e.g. Clarkson *et al.* 1992). The adaptation which reduces muscle injury in a second eccentric exercise bout can occur within five days (Ebbeling & Clarkson 1990) and can be induced by 10 eccentric muscle actions (Brown *et al.* 1996). The mechanisms underlying this rapid and long lasting adaptation are poorly understood (Clarkson *et al.* 1992). Saxton & Donnelly (1996) reported changes in isometric force consistent with an increase in ML following eccentric exercise. Lynn & Morgan (1994) proposed the adaptation resulted from an increase in resting muscle length, shifting the length tension curve to the left and thereby reducing sarcomere stretching. In the present study MVC measurements were collected over the time course during which Ebbeling & Clarkson (1990) reported muscle adaptation occurred. Furthermore, Brown *et al.* (1996) have reported a prophylactic effect to eccentric exercise induced muscle injury using a similar exercise regimen to that of the current study. As the increase in muscle length was transient in this investigation and adaptation lasts for several weeks (Brown *et al.* 1996), there must be at least one other factor which attenuates injury when performing subsequent eccentric exercise.

Performing high force eccentric actions at a long muscle length may provide an efficient method of inducing micro damage to the sarcomere structure. As such damage is thought to provide the stimulus for repair and compensatory growth (Goldspink 1971, Taylor & Wilkinson 1986), the findings of this study may have practical implications for both training and sports performance. When attempting to increase muscle mass eccentric actions at long muscle length may maintain the stimulus for growth with a reduced training volume. The loss of proprioception following eccentric exercise (Saxton *et al.* 1995), and angle specific force deficits (Saxton & Donnelly 1996) also observed in this study may require several days to recover. Therefore, following eccentrically biased training sufficient recuperation should be provided to allow optimal performance in competition, especially if the sport demands forceful or precise movements.

The initial event in eccentric exercise induced muscle damage appears to be mechanical in origin although the mechanism of injury is still unclear. This study provides support for Morgans' (1990) 'sarcomere popping' theory of damage, but there is no evidence to suggest the prophylactic effect of eccentric exercise results from an increase in ML.

# CHAPTER 4

## Manipulation of knee extensor force using percutaneous electrical myostimulation during eccentric actions, effects on indices of muscle damage in humans.

Aspects of this study were presented to the Physiological Society, Birmingham, UK (1995).

This study has been published in abstract form (R.B. Child, S.J. Brown, A.E. Donnelly, J.M. Saxton, S.H. Day 1995 Effects of stimulated eccentric muscle actions at 20Hz and 100Hz on indices of muscle damage in man. Journal of Physiology 483: 129-130.

**Summary**

Percutaneous electrical myostimulation (PES) was used to manipulate the force produced by the knee extensor muscles during eccentric exercise, thereby providing a model to investigate the role of force in muscle damage. Two eccentric exercise bouts of equal work were performed by nine subjects (5 male, 4 female), using fixed voltage PES at 20Hz (to produce moderate muscle forces) and 100Hz (to produce high muscle forces). Muscle contractility, serum creatine kinase activity (CK) and muscle soreness (MS) were analysed before and up to 14 days after exercise. Data were analysed using repeated measures analysis of variance (ANOVA), *t*-tests and Wilcoxon tests. Peak forces were different during the first 60 repetitions of each bout ($P<0.001$, ANOVA), with higher forces during the 100Hz bout for repetitions 1, 10, 20 ($P<0.01$, *t*-test) and 30 ($P<0.05$, *t*-test). Following the 100Hz bout, maximum voluntary contractile force was lower ($P<0.01$, ANOVA), and CK was higher ($P<0.0001$, ANOVA) than after 20Hz bout. Subjects reported greater MS on days 2 to 6 ($P<0.05$, Wilcoxon test) following the 100Hz bout. Despite a decline in the 20:100 force ratio after each bout ($P<0.01$, ANOVA) there was no difference between bouts ($P>0.05$, ANOVA). All indirect markers of muscle injury suggested both exercise bouts resulted in muscle damage. The greater magnitude of change for maximum voluntary contractile force, CK and MS after the 100Hz bout suggest the force per fibre contributes to muscle damage.

## Introduction

The initial event in eccentric exercise induced injury appears to be mechanical in origin (McCully & Faulkner 1986, Friden & Lieber 1992, Warren *et al.* 1993). Generation of high forces in active fibres has been proposed as a factor in the process of muscle injury (Katz 1939, Friden *et al.* 1983, Armstrong *et al.* 1991). To date the force generated per fibre has not been altered independently of other mechanical factors in humans and the role of force in muscle injury is still debated (Jubrias & Klug 1993).

Lieber & Friden (1993) concluded muscle damage was not a function of force but active muscle strain, however an equally convincing body of literature has shown force to be important in muscle damage (McCully & Faulkner 1986, Warren *et al.* 1993). These studies were conduced using a variety of animal species, which may explain the disparate conclusions. Alternatively the diverse experimental protocols used to induce damage might also explain these conflicting reports (Faulkner *et al.* 1993). Clearly some caution must be used when extending to humans, insights obtained in animal experiments.

The use of PES during eccentric actions provides a novel method to manipulate the force per fibre in human muscle. Using this exercise model the potential difference or 'excitatory stimulus' and frequency of stimulation (i.e. the number of pulses delivered per second) can be altered to control the muscle force generated (Edwards *et al.* 1977, Robinson & Snyder-Mackler 1995). The frequency of stimulation determines the number of fibres which reached fused tetanus. By maintaining a constant potential difference for each pulse and altering the stimulation frequency, varying forces can be produced within a fixed mass of muscle (Robinson & Snyder-Mackler 1995). Isokinetic exercise in combination with PES allows force to be controlled independently of muscle strain. This study aimed to investigate the effect of fibre force during eccentric exercise on immediate and delayed indices of muscle damage in humans.

## Methods

*Subjects*

Study approval was obtained from Wolverhampton University ethics committee, and all volunteers completed informed consent documents. Untrained volunteers 5 male, 4 female (age range 18 to 35 years) completed two bouts of eccentric exercise, randomised for order effects. Each bout was separated by a two week recovery period. In one bout PES was applied to the knee extensors (KE) at 20Hz; in the other PES was applied at 100Hz to the contralateral KE.

*Percutaneous electrical myostimulation (PES)*

Knee extensor muscles were stimulated using the protocol and equipment described in section 2.3.

*Isometric force measurement*

Knee extensor (KE) force was measured in a seated position at a knee flexion angle of 1.57 rad. using the strain gauge described in section 2.2. This system was used in preference to the Kin-Com for measurements of isometric force due to higher signal sampling frequency and the ease at which subjects could be secured in order to isolate the KE. After familiarisation MVC, MVS and the 20:100 force ratio were measured as described in section 2.3. These measurements were made prior to and 5 minutes after exercise; and then again on days 1, 2, 3, 7 and 9 subsequent to each bout.

*Exercise*

Eccentric actions were produced solely by the application of PES. These were performed on an isokinetic dynamometer (described in section 2.1) using stimulation frequencies of 20 and 100Hz in each respective

exercise bout. The contralateral KEs were used in the second bout of exercise which was performed two weeks after the first.

For exercise at 100Hz the stimulation voltage (set prior to the bout) was sufficient to induce 50% of maximum voluntary contractile force (MVC) at a knee angle of 1.57 rad. at 100Hz. For the 20Hz bout volunteers were again stimulated to 50% MVC using the same protocol, but stimulation frequency was reduced to 20Hz for the exercise bout. A preload was used such that the knee extensors were stimulated for 0.5 seconds before the onset of movement. This allowed isometric force to rise prior to movement of the dynamometer lever arm. Each eccentric action was performed through a 1.57 rad. knee joint range of motion (from 2.96 rad. to 1.39 rad.) at an angular velocity of 1.05 $rad.s^{-1}$. Repetitions were separated by an 8 seconds rest period, during which the leg was returned passively by the experimenter at 1.05 $rad.s^{-1}$.

Peak force and work were determined for every tenth repetition during exercise. Peak force achieved during each eccentric action was measured directly from the force trace. The work performed in each stretch was calculated from the average force exerted and the perpendicular distance through which it was applied. Total work was monitored during each bout and the number of repetitions performed by each subject were varied to balance the total work.

*Serum creatine kinase activity (CK)*

Blood samples were drawn as described in section 2.5, pre-exercise, then again on days 1, 2, 3, 7 and 9 after exercise. Serum samples were analysed for creatine kinase activity, as described in section 2.6.

*Muscle soreness*

Soreness was assessed before exercise and on each subsequent day at 8 muscle regions (6 extensor 2 flexor) depicted on a questionnaire (Appendix 1). Soreness was determined using the protocol described in section 2.4. Each subjects soreness values for the 8 muscle sites were summed on each day, and the total used as the criterion score.

*Statistics*

Statistical analyses were performed as described in section 2.13, with all data presented as means±SEM.

## Results

*Exercise*

Original force records (n=1) for the first and final repetitions of the exercise bouts performed using 20Hz and 100Hz PES are shown in Fig. 4.1. A much greater decline in peak force and total work occurred following the 100Hz bout than the 20Hz bout.

Changes in peak force during eccentric exercise are shown in Fig. 4.2. Peak force was different between bouts during the first 60 repetitions ($P<0.001$, ANOVA). Post hoc comparisons showed higher peak forces during the 100Hz bout for repetitions 1, 10 and 20 ($P<0.01$, $t$-test) and 30 ($P<0.05$, $t$-test). By repetition 40 peak force was not significantly different between bouts ($P>0.05$, $t$-test). For 20 and 100Hz bouts total work was 4609±796 and 4671±553 Joules respectively ($P>0.05$, $t$-test). To achieve this a total of 106±6 and 96±15 repetitions were performed in 20 and 100Hz bouts respectively ($P>0.05$, $t$-test).

To provide an index of the force produced by each muscle fibre during the stimulated eccentric exercise bouts, peak force was normalised relative to the MVC produced prior to eccentric exercise (Table 4.1).

*Isometric force*

Changes in isometric force in response to eccentric exercise bouts are shown in Fig. 4.3 . The pre-exercise values for contractile parameters were not different between bouts ($P>0.05$, $t$-test). Before exercise superimposed stimulation failed to increase knee extensor force ($P>0.05$, $t$-test), where mean values were 360±49N and 356±53N for MVC and MVS respectively.

After 100Hz exercise MVS was reduced ($P<0.001$, ANOVA), with lowest force recorded on day 3. MVS was below baseline levels post-exercise; on days 1, 2, 3 ($P<0.01$, Newman-Keuls test), and on days 7 and 9 ($P<0.05$, Newman-Keuls test). Although MVS was lower after 20Hz exercise ($P<0.001$, ANOVA) it was not different from pre-exercise values at any time point ($P>0.05$, Newman-Keuls test).

After 20Hz exercise MVS was higher than after the 100Hz bout ($P<0.01$, ANOVA). Between bout comparisons revealed lower MVS after the 100Hz bout post-exercise, on day 2 ($P<0.05$, $t$-test) and days 3, 7 and 9 ($P<0.01$, $t$-test). Superimposed stimulation failed to increase maximum KE force after 20Hz exercise. In contrast after 100Hz exercise the MVS was higher than the MVC ($P<0.05$, ANOVA), on days 1, 2 and 3 ($P<0.05$, $t$-test). Therefore MVS measurements after the 100Hz bout reflect the KE muscles force generating potential more accurately than the MVC.

*20:100 Force ratio*

Changes in the 20:100 force ratio in response to eccentric exercise bouts are shown in Fig. 4.4. A decline in 20:100 force ratio was observed after both exercise bouts ($P<0.01$, ANOVA), although no difference was observed between exercise bouts ($P>0.05$, ANOVA).

*Creatine kinase*

Changes in CK in response to eccentric exercise bouts are shown in Fig. 4.5. CK differed between bouts ($P<0.0001$, ANOVA); $t$-tests established greater activity on day 1 ($P<0.01$), day 2 ($P<0.001$), day 3 ($P<0.0001$), day 7 ($P<0.001$) and day 9 ($P<0.001$) after the 100Hz bout.

CK was different from baseline after the 100Hz bout ($P<0.0001$, ANOVA), post hoc tests established elevations on days 1, 2, 3 and 7 ($P<0.01$, Newman-Keuls test) and day 9 ($P<0.05$, Newman-Keuls test). After the 20Hz bout CK was different from baseline ($P<0.0001$, ANOVA), post hoc comparisons revealed significant elevations on day 1 ($P<0.05$, Newman-Keuls test) and days 2 and 3 ($P<0.01$, Newman-Keuls test).

*Soreness*

Changes in muscle soreness in response to eccentric exercise bouts are shown in Fig. 4.6. Muscle soreness was reported on days 1 to 5 after

the 20 Hz exercise and days 1 to 7 after 100Hz exercise ($P<0.05$, Wilcoxon test). Soreness was higher relative to the 20Hz bout on days 2 to 6 after 100Hz exercise ($P<0.05$, Wilcoxon test), with peak soreness observed on days 2 and 3 after each respective bout. No soreness was observed in the knee flexor muscles after exercise.

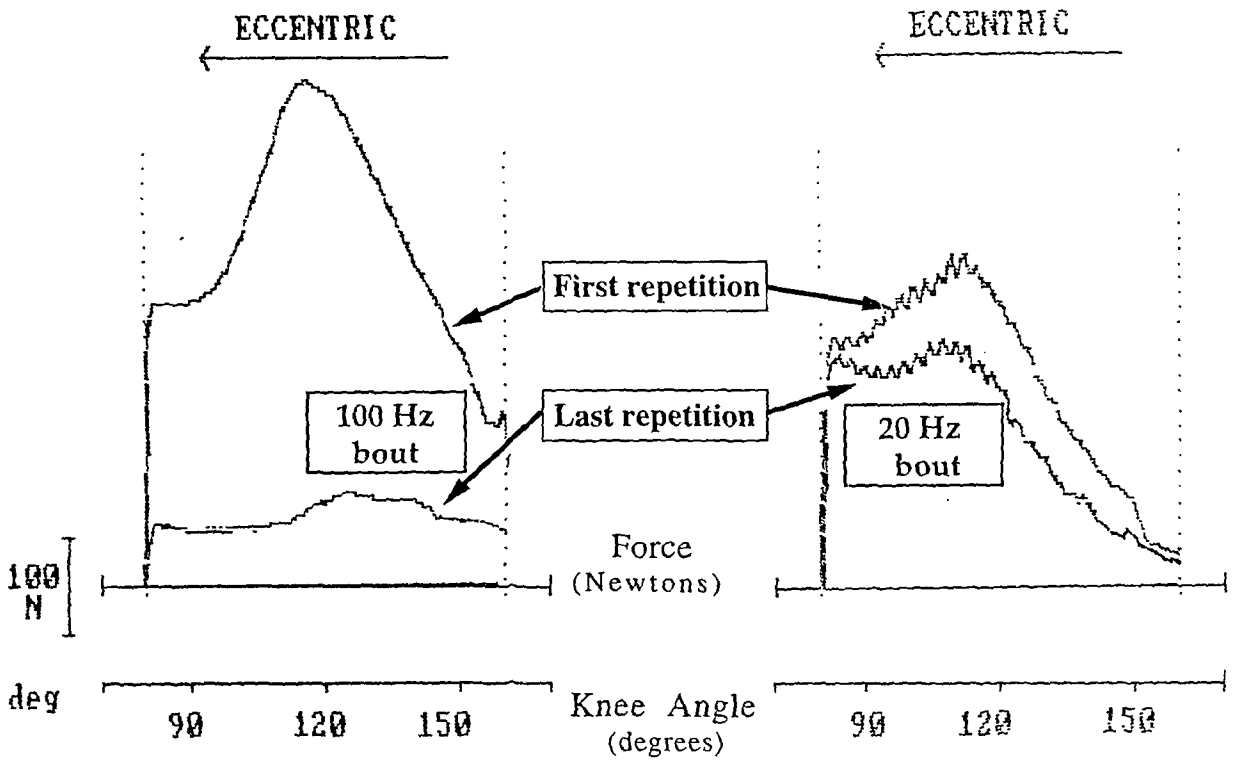


**Fig. 4.1 Typical force traces produced using PES during eccentric actions.**

Note values are only reported for one subject.

**Table 4.1 Peak eccentric force normalised as a percentage of the pre-exercise isometric MVC.**

| | Repetition number | | | | | | |
|---|---|---|---|---|---|---|---|
| **Exercise** | Rep. 1 | Rep. 10 | Rep. 20 | Rep. 30 | Rep. 40 | Rep. 50 | Rep. 60 |
| 20Hz bout | 58% (5) | 57% (6) | 54% (5) | 50% (5) | 47% (5) | 46% (5) | 42% (4) |
| 100Hz bout | 125% (15) | 95% (9) | 78% (10) | 76% (11) | 61% (9) | 55% (8) | 55% (10) |

Note As only half the KE muscle mass was active during each exercise bout the generation of 50% or more of the isometric MVC represents supermaximal force generation from the active muscle fibres.

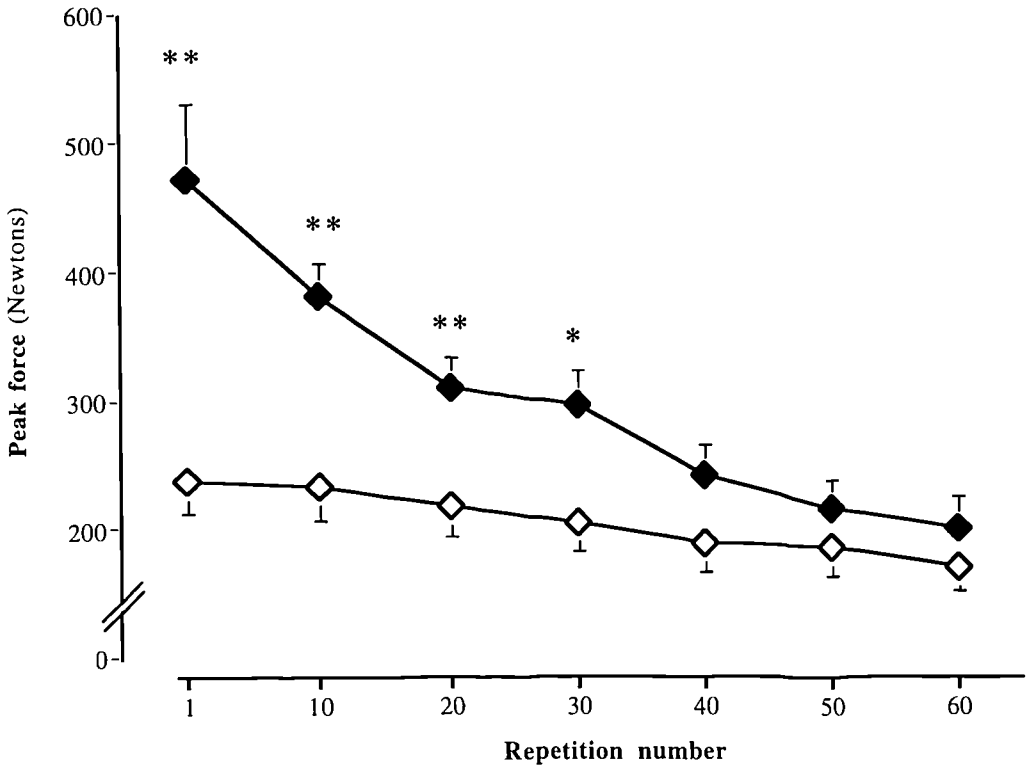


**Fig. 4.2. *Changes in peak force during the first 60 repetitions* of exercise bouts at 20Hz (open diamonds) and 100Hz (filled diamonds).**

As a consequence of the randomised study design it was only possible to perform paired comparisons for the minimum number of eccentric repetitions performed in a single exercise bout i.e. 60. The difference in the total number of repetitions performed between bouts for individual subjects arose from the need to balance the total eccentric work performed. ** $P<0.01$, * $P<0.05$, *t*-test, between bouts.

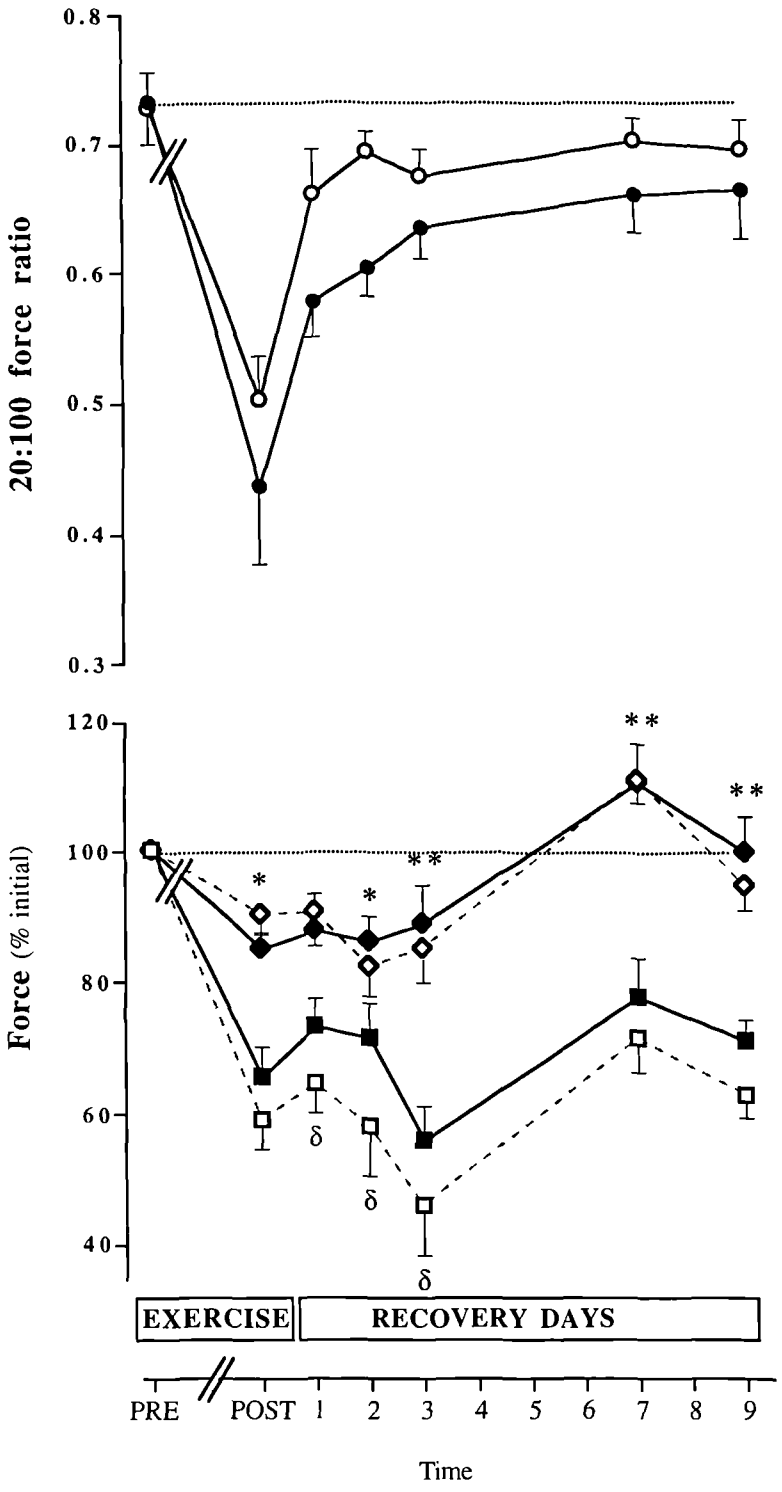


**Fig. 4.3** (*Top*) **Changes in the 20:100 force ratio following exercise bouts at 20Hz (open circles) and 100Hz (filled circles).** (*Bottom*) **Changes in maximal voluntary contractile force (MVC) following exercise bouts at 20Hz (open diamonds) and 100Hz (open squares). Changes in maximal voluntary contractile force with PES (MVS) following exercise bouts at 20Hz (filled diamonds) and 100Hz (filled squares).** * $P<0.05$ between bouts, ** $P<0.01$ between bouts, δ $P<0.05$ MVS higher than MVC after 100Hz exercise.

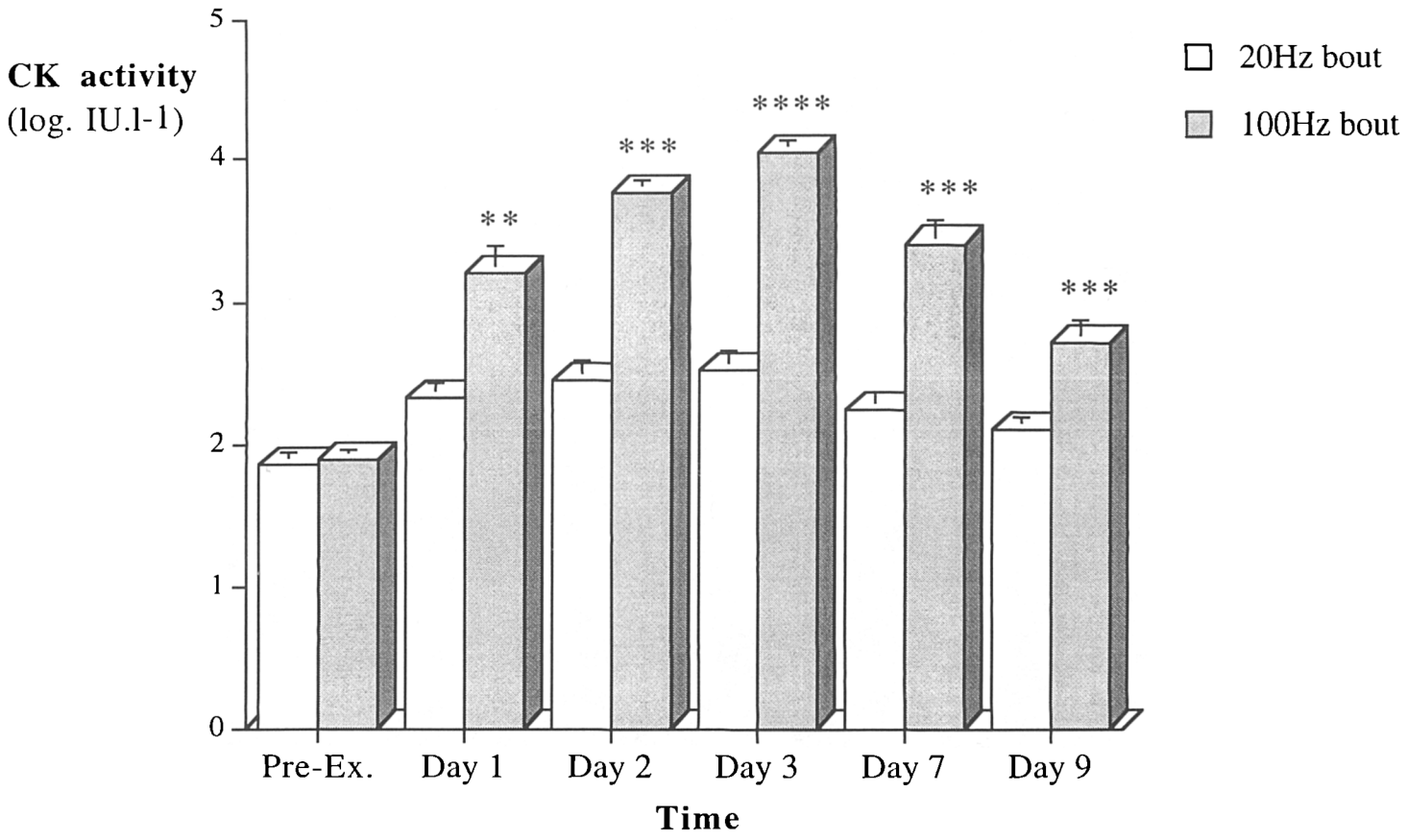


**Fig 4.4 Changes in serum creatine kinase activity following stimulated exercise bouts at 20Hz and 100Hz.** $^{**}P<0.01$, $P<0.001$, $P<0.0001$, between bouts.

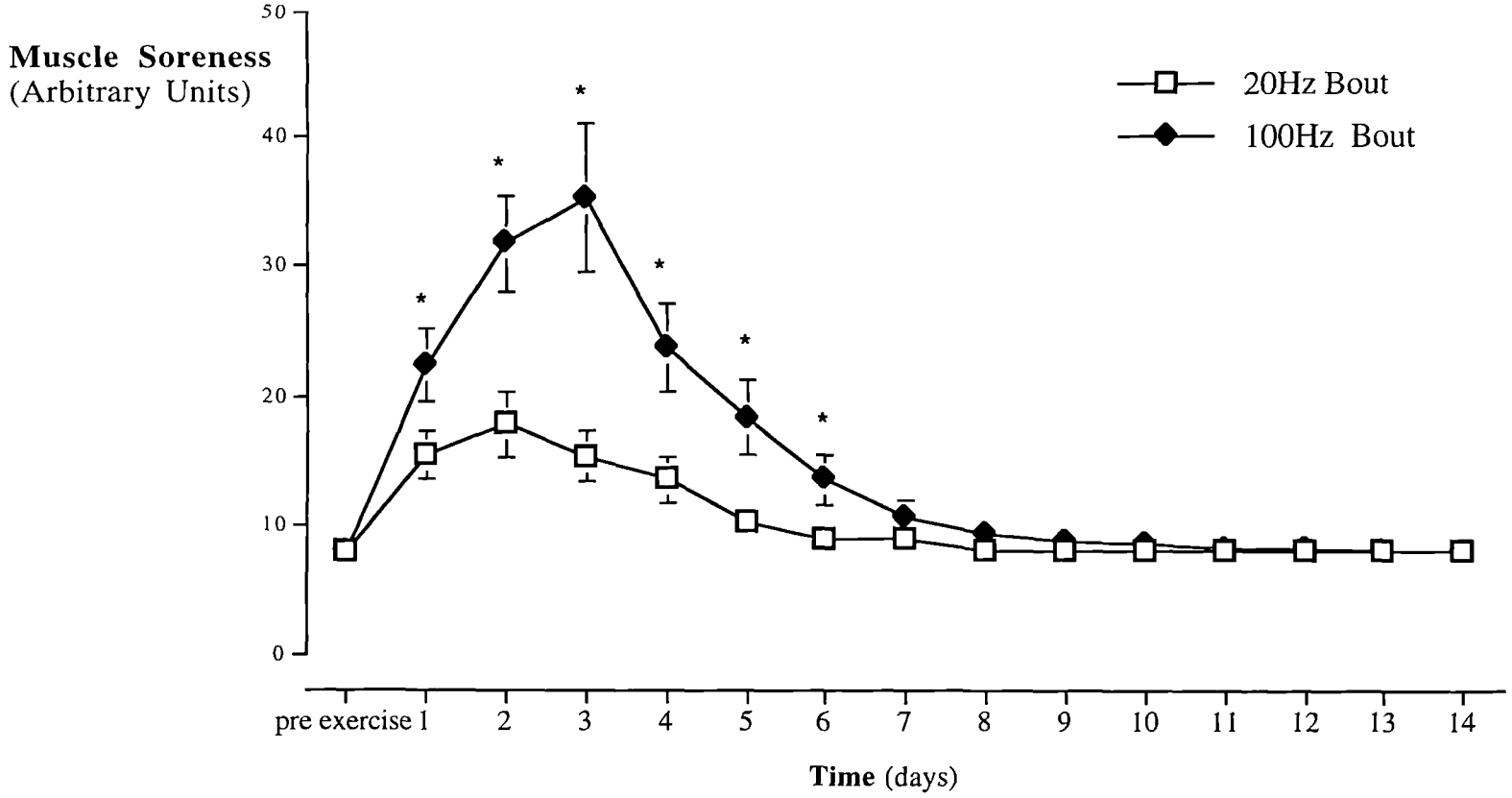


**Fig. 4.5 Changes in muscle soreness following stimulated exercise bouts at 20Hz and 100Hz.**

Soreness values are means for 8 muscle sites (6 extensor, 2 flexor). This gives a value of 8 for no soreness, with a maximum soreness of 80. * $P<0.05$, Wilcoxon test, between bouts.

## Discussion

All subjects successfully completed both exercise bouts in which strain rate and magnitude were identical, and total eccentric work was equally matched within subjects.

Isometric force measurements using PES would suggest approximately 50% of the KE musculature were activated by PES. Therefore in some regions of the muscle the applied potential difference was insufficient to produce depolarisation of the total fibre population. In these domains type II fibres may be preferentially recruited, as a consequence of their lower activation threshold, when applying PES (Robinson & Snyder-Mackler 1995). This partial modification of muscle fibre recruitment patterns may not threaten the external validity of the study as similar changes are thought to occur when performing voluntary eccentric actions (Nardone & Schieppati 1988, Nardone & Romano 1989).

The decline in force during both exercise bouts (Fig. 4.1 and Fig. 4.2) probably reflects the combined effects of muscle damage and fatigue. At the end of the 100Hz bout forces were lower than at the end of the 20Hz bout (Fig. 4.1). McCully & Faulkner (1986) showed activation of fatigued fibres during eccentric actions does not result in injury, possibly as a consequence of the lower forces produced in the fatigued state. Therefore it is plausible the damage which resulted from exercise in this study occurred in the earlier repetitions of both exercise bouts, when highest forces were generated (Fig. 4.2).

Following exercise, fatigue could contribute to the post-exercise decline in MVS (Fig. 4.3), although a day or more after exercise complete recovery from fatigue mediated force losses would be expected (Edwards *et al.* 1977, Fitts 1994). When recovery from fatigue is complete the deficit in maximum isometric force is thought to be the most valid measure for the totality of muscle damage (Warren *et al.* 1993, Faulkner *et al.* 1993, Brooks *et al.* 1995). If this is the case the lower MVS may indicate the magnitude of muscle injury was greater after the 100Hz bout.

Clarkson *et al.* (1992) have reported a continuous recovery of force in the days following exercise, however in this study minimum force was recorded 2 and 3 days after exercise for 20 and 100Hz

exercise bouts respectively. Delayed decrements in maximum force loss have also been seen in man (Jones *et al.* 1989) and mice (McCully & Faulkner 1986, Zerba *et al.* 1990) following eccentric work. Such functional evidence for delayed damage may be related to histological evidence of increased myofibrillar disruption at similar time points post exercise (Newham *et al.* 1983b, Friden *et al.* 1983).

The increase in isometric force when applying PES to the MVC is a novel finding, and suggests myostimulation may have overridden the mechanisms limiting force production. Previous researches have not observed this effect when applying PES to damaged muscle (Jones *et al.* 1989, Newhan *et al.* 1987) and there are several possibilities which may explain this phenomenon. Damage might have been more extensive following the 100Hz exercise, which may have resulted in a larger population of motor units which could not be activated voluntarily. Alternatively, stimulation of a larger proportion of the total muscle mass than achieved by previous investigators (e.g. Rutherford *et al.* 1986), may have recruited a larger number of the fibres from the inactive pool.

If maximum force production is neurally limited it may be related to sensitisation of type III and IV muscle afferents. Not only are these nerves involved in the perception of soreness (Hayward *et al.* 1991), they may also have the potential to modify motor output (Bigland-Ritchie *et al.* 1986, Hayward *et al.* 1991). There may be several advantages to limiting the generation of high force in damaged muscle. It has been proposed the inhibition of activation at the first step of excitation contraction coupling prevents initiation of subsequent ATP utilising steps and increases in calcium (Fitts 1994). Such a mechanism may also protect vulnerable motor units (i.e. those which are damaged or undergoing repair) from further mechanically or metabolically induced damage.

As peak force was the only factor which differed between bouts in this study, it would appear initial muscle injury resulted from the higher forces generated in the earlier repetitions of the 100Hz bout. Thus the findings of the current investigation suggest force is an important factor in the initiation of muscle injury in humans. Possible mechanisms of force induced muscle injury are discussed in several reviews (Stauber 1989, Armstrong *et al.* 1991, Friden & Lieber 1992). Hasselman *et al.* (1995) proposed the concept of a force threshold for damage, above

which sequential disruption occurred to the myofibrils and cytoskeleton. If such a threshold exists in humans it would appear to be close to the sarcomere force produced during maximal isometric contractions, as such contractions produce evidence of muscle damage (Talag 1973, Jones *et al.* 1989, Kroon & Naeije 1991). Estimates of the force produced during eccentric exercise in the muscle recruited by PES might be analogous to the force produced in the active fibres. Relative force estimates (Table 4.1) suggest the force per fibre was above that produced during MVCs, and therefore the threshold force for damage. The post-exercise changes in all damage indices also suggest the force threshold for damage was exceeded in both exercise bouts.

The presence of LFF can provide evidence for muscle damage (Edwards *et al.* 1977, Jones *et al.* 1986). Despite the decline in the 20:100 force ratio after exercise no difference was observed between bouts (Fig. 4.3). This suggests LFF was independent of the forces generated during eccentric contractions, as originally proposed by Jones *et al.* (1989).

It is unclear from previous studies how the tension produced during eccentric exercise affects indices of secondary damage (Warren *et al.* 1993), which may include increased myocellular proteins in serum and MS (Faulkner *et al.* 1993). The delayed elevation in CK commonly observed following eccentric exercise in humans (Jones *et al.* 1986, Newham 1988), may represent combined membrane damage and muscle fibre necrosis (Evans & Cannon 1991, Armstrong *et al.* 1991, Newham *et al.* 1983a). The greater rise in CK after 100Hz exercise suggests a larger number of fibres either sustained sublethal injury or became necrotic. Whichever is the case, this study provides evidence that the elevation in CK (Fig. 4.4) and MS (Fig. 4.5) were dependent on the forces produced during exercise. Warren *et al.* (1993) reported creatine kinase release was independent of the force produced by rat *soleus* muscle, in the 1 hour period following eccentric exercise. Such observations are consistent with the myocellular release of creatine kinase from progressive degradation of damaged fibres, rather than membrane rupture during the exercise bout.

This study attempted to manipulate the force per fibre independently of muscle strain and the total eccentric work performed during each eccentric exercise bout. The experimental findings suggest within the range of strains encountered *in vivo*, force is an important

factor in the initiation of muscle injury in humans. The large post-exercise difference in MVS, CK and MS between bouts indicate the forces generated during eccentric actions contribute to secondary muscle injury .

# CHAPTER 5

## Changes in indices of antioxidant status, lipid peroxidation, and inflammation, in human skeletal muscle following damaging eccentric exercise.

Aspects of this study were presented at the 1st European Sports Science Congress, Nice, France (1996) and to the London meeting of the Physiological Society (1996) and have been published in abstract form (R.B. Child, S.J. Brown, S.H. Day, A.E. Donnelly, H. Roper, J.M. Saxton 1996 Elevated muscle β-glucuronidase and G6PDH activity as biochemical markers of exercise induced muscle damage in humans. Journal of Physiology 494: 132).

I gratefully acknowledge the work of Dr. John Saxton in sectioning and staining the muscle biopsy specimens.

## Summary

This study investigated the effects of muscle inflammation on indices of antioxidant status and muscle injury following eccentric exercise. Eight subjects each performed 70 maximal voluntary eccentric muscle actions, with myostimulation superimposed. Exercise was performed on an isokinetic dynamometer, using the knee extensors of a single leg. Venous blood samples were collected into plain and EDTA tubes 5 and 3 days before exercise, immediately before exercise, and then again on days 3, 4, 5, 6, 7, 10 and 12 following the bout. Needle biopsies (*vastus lateralis*) were taken from 6 subjects a week prior to exercise (baseline) and again on days 4 and 7 after exercise. The malondialdehyde concentration in plasma and muscle were used as markers of lipid peroxidation. Creatine kinase activity, β-Glucuronidase activity (βG) and total antioxidant capacity (TAC) were determined in serum. In muscle, aqueous and bound TAC, aqueous sulphydryl concentration (SH), βG and Glucose-6-phosphate dehydrogenase activity (G6PDH) were determined.

Biochemical data were analysed using repeated measures analysis of variance (ANOVA). No changes were observed in serum TAC, although activities of creatine kinase and β–Glucuronidase were elevated ($P<0.05$). In muscle, aqueous and bound TAC, SH, G6PDH and βG were all elevated ($P<0.05$). As the malondialdehyde concentration was unaltered, this study gives no support for free radical injury resulting from muscle inflammation.

## Introduction

Initial muscle injury during high force eccentric actions appears to be mechanically mediated (McCully & Faulkner 1986, Armstrong *et al.* 1991, Friden & Lieber 1992, Warren *et al.* 1992, Chapters 3 & 4). Faulkner *et al.* (1993) proposed the delayed rise in CK and MS may be manifestations of secondary damage. This appears to have metabolic origins (Armstrong *et al.* 1991, Duncan & Jackson 1987) and several reports have implicated free radicals in the damage process (Zerba *et al.* 1990, Warren *et al.* 1992, Duarte *et al.* 1994). Following initial injury radical oxidative stress (ROS) could be elevated by the presence of neutrophils and macrophages in muscle. The production of free radicals by such infiltrates could damage lipid membranes, resulting in cell necrosis (Kramer *et al.* 1984, Halliwell & Chirico 1993). Free radical mediated muscle injury, resulting from inflammation, has been used to explain several muscle damage phenomena. Warren *et al.* (1992) proposed free radical damage might be responsible for the delay in force loss, sometimes observed following eccentric exercise (Zerba *et al.* 1990, Chapter 4); while Cannon *et al.* (1990) suggested free radicals from cellular infiltrates could result in delayed elevations in CK.

Theoretical models to explain various aspects of secondary muscle damage have relied heavily on findings from experiments using rodents (e.g. Salminen & Vihko 1983, Zerba *et al.* 1990, Duarte *et al.* 1994). Faulkner *et al.* (1993) concluded 'the mouse model of contraction induced injury provides an accurate replica of the human model.' This is unlikely as there are many species differences which are of importance when extrapolating experimental findings to humans. These include the damage susceptibility of fibres (Friden *et al.* 1983, Armstrong *et al.* 1983), fibre oxidative capacity (Holloszy & Booth 1976), inflammatory response (Weiss 1989) and the time course for regeneration (Jones *et al.* 1986, Salminen & Kihlstrom 1985). Many animal studies investigating exercise myopathy have used treadmill running to produce tissue injury (e.g. Salminen & Vihko 1983, Duarte *et al.* 1994), which inherently increases free radical activity in the muscle (Davies *et al.* 1982). This may be a consequence of the high oxidative demands of such activity and the associated formation of reactive oxygen species by muscle mitochondria (discussed in section

1.0.4). This may make changes in free radical indices difficult to interpret, especially in the early stages of exercise recovery (Witt *et al.* 1992). In an attempt to isolate the effects of ROS resulting from muscle damage, from the metabolic disturbances which occur following whole body activity, an exercise protocol with a low metabolic demand was utilised.

The mechanism underlying free radical mediated tissue injury may involve increases in ROS overwhelming antioxidant defenses (Halliwell & Chirico 1993). Malondialdehyde (MDA) has been used as a marker of peroxidative damage in several studies of inflammatory myopathy (Salminen & Vihko 1983, Burr *et al.* 1987, Asayama *et al.* 1989). To the authors knowledge, the simultaneous assessment of free radical protection in muscle has not been performed, although such an approach may help to elucidate the possible role of free radicals in exercise myopathy. When assessing antioxidants individually, it is necessary to determine the concentration of a spectrum of compounds to provide an index of total free radical protection. Developments in assay techniques now allow the total free radical quenching capacity of complex antioxidant solutions to be quantified. The method of Whitehead *et al.* (1992) has proved effective in detecting reduced serum total antioxidant capacity (TAC) in disease states associated with increased free radical stress (Whitehead *et al.* 1992).

Indices of free radical damage in human muscle have previously been evaluated following eccentric and concentric exercise (Saxton *et al.* 1994). These authors found no evidence that eccentric exercise resulted in free radical damage. However, there was also no evidence that the exercise bout produced mechanically mediated muscle injury, or resulted in muscle inflammation. These events may be essential pre-requisites for free radical mediated muscle damage to occur following eccentric actions. The purpose of this study was to determine if inflammatory processes and cellular infiltration, contributed to the delayed release of creatine kinase, by compromising antioxidant defenses and promoting lipid peroxidation. Muscle inflammation was assessed biochemically and histologically. Muscle MDA content was used as a marker of lipid peroxidation, with muscle antioxidant capacity and sulphydryl content as indices of free radical protection.

## Methods

*Subjects*

Eight physically active but untrained volunteers (4 male and 4 female, aged 21 to 31 years) participated in the study, which was approved by Wolverhampton University Ethics Committee.

*Percutaneous electrical myostimulation (PES)*

Knee extensor muscles were stimulated using techniques described in section 2.3. Using a stimulation frequency of 100Hz the voltage used for PES was sufficient to elicit 30% of the maximum voluntary contractile force at a knee angle of 1.92 rad. on the day of testing.

*Isometric knee extensor (KE) force measurement*

Force measures were performed in duplicate on a isokinetic dynamometer (described in section 2.1). Maximum knee extensor contractile force with PES (MVS) and the 20:100 force ratio were determined as described in section 2.3. Isometric KE force measurements were determined at a knee angle of 1.92 rads. prior to and 5 minutes after exercise.

*Exercise*

Each subject performed 70 maximal voluntary eccentric muscle actions on a Kin-Com isokinetic dynamometer, using the knee extensors of a randomly selected leg. Exercise was performed in a prone position using a knee joint range of motion from almost full extension to almost full flexion. A preload was used so that each subject produced maximal knee extensor force prior to movement of the dynamometer lever arm; this allowed isometric force to rise before the eccentric action. PES

(using identical settings to those for the pre-exercise MVS) was applied for 0.5 seconds prior to movement of the lever arm and throughout the eccentric action. Each eccentric action was performed at an angular velocity of 1.75 rad.s$^{-1}$. Each repetition was separated by a 10 second rest period, during which the leg was returned to the start position by the experimenter at 1.05 rad.s$^{-1}$.

*Blood sampling*

Ten milliliter blood samples were drawn from an antecubital vein (as described in section 2.5), 5 days, 3 days and immediately pre-exercise, then again on days 3, 4, 5, 6, 7, 10 and 12 after the exercise bout.

*Biopsy collection and preparation*

Under local anaesthesis (2% lignocane) biopsies were removed using a Berstrom-type needle (diameter 6.0 to 6.5mm). Each sample was taken from the distal region of the *vastus lateralis* using a slightly different location on each occasion. Baseline biopsies were taken 4 days before exercise from a randomly selected leg, with further samples from the exercised leg 4 and 7 days after the bout. Each biopsy sample was quickly divided for histological and biochemical analysis. Tissue for biochemical analysis was frozen in liquid nitrogen, while tissue for histological analysis was frozen in isopentane (chilled in liquid nitrogen). Tissue samples were then stored at -80$^{o}$C until analysis.

*Histological analysis*

Biopsy specimens were divided for evaluation of pathological changes using light microscopy (LM) and ultrastructural changes using electron microscopy (EM). The evaluation of ultrastructural changes is the subject of another investigation which will be presented in a future study. Specimens prepared for LM were mounted in blocks in a cryostat. Transverse sections (5 to 6 microns thick) were cut at -20$^{o}$C, mounted on glass slides, air dried and stained with hematoxylin-eosin.

*Biopsy preparation*

After dissecting the muscle free of visible connective tissue and fat, it was homogenised in degassed ice cold 50 mmol. phosphate buffer at pH 7.4 (approximately 10% weight for volume). The homogenate was then transferred to a pre-weighed vial and centrifuged at 13,000*g* for 10 minutes at 3°C. The supernatant was divided into aliquots, frozen in liquid nitrogen and stored at -80°C. After removal of the remaining supernatant, the pellet was washed twice in ice cold 50 mmol phosphate buffer at pH 7.4 . Each wash consisted of re-suspending the pellet in 1 ml of buffer, vortexing for 1 minute (to form a milky solution), centrifugation at 13,000*g* and removal of the supernatant layer. The pellet was then frozen in liquid nitrogen, freeze dried at -20°C for 5 hours after which the combined weight of the vial and pellet were noted. The pellets were stored in sealed vials at -80°C for 4 days prior to analysis.

*Serum creatine kinase activity (CK)*

Serum creatine kinase activity was determined using the protocol described in section 2.6.

*β-Glucuronidase activity (βG)*

The activity of β-Glucuronidase was determined using the protocol described in section 2.8.

*Glucose 6 phosphate dehydrogenase activity (G6PDH)*

Muscle G6PDH was determined using 25μl of muscle homogenate, following the procedure described in section 2.7.

*Serum uric acid concentration*

The serum uric acid concentration was determined using the protocol described in section 2.9.

*Antioxidant capacity (TAC)*

Serum antioxidant capacity was evaluated using the protocol described in section 2.10. Aqueous muscle antioxidants were determined without further preparation. Antioxidant capacity for the biopsy pellet was determined after re-weighing (to determine there was no moisture uptake). The pellet was then re-suspended in 200ml.g$^{-1}$ dimethyl sulphoxide (DMSO) at room temperature, vortexed for 5 minutes and centrifuged at 13,000*g* for 5 minutes. The supernatant layer was carefully removed for analysis without further preparation. The homogenisation buffer, DMSO and serum diluant were taken from the same stock and verified to have no antioxidant properties (within the detection limits of the assay) prior to and following sample analysis.

*Total sulphydryls*

The concentration of aqueous sulphydryls in muscle were determined using the protocol described in section 2.11.

*Malondialdehyde (MDA)*

The concentration of MDA was determined using the protocol outlined in section 2.12.

*Muscle soreness (MS)*

Soreness was assessed before exercise and on each subsequent day at 8 muscle regions (6 extensor 2 flexor) depicted on a questionnaire (Appendix 1). Soreness evaluation was performed as described in

section 2.4. Each subjects soreness values (for the 6 KE muscle sites) were summed each day, and the total used as the criterion score.

*Statistics*

Statistical analysis were performed as described in section 2.13. Muscle TAC, MDA concentration and enzyme activities were analysed following logarithmic transformation to minimise variance heterogeneity over the time course of the study. These data are presented in table 5.2, with same data prior to logarithmic transformation presented in Appendix 8. Blood and functional data are for n=8 subjects while muscle biochemical data are for n=6 subjects. All biochemical and functional data are presented as means with the SEM. Soreness data are presented as means with the range in parenthesis.

## Results

*Isometric force*

Exercise resulted in a decrease in MVS from 259±25 Nm to 131±12 Nm ($P<0.001$, *t*-test). The 20:100 force ratio decreased from 0.74±0.03 to 0.29±0.04 ($P<0.001$, *t*-test).

*Muscle Soreness*

Soreness of the KE differed from pre-exercise values between days 2 and 6 following the bout ($P<0.05$, Wilcoxon test).

*Blood*

CK activity did not differ between the baseline biopsy and pre-exercise, however following exercise CK was different from baseline ($P<0.0001$, ANOVA). Newman-Keuls tests established elevations on days 3, 4 and 5 after exercise ($P<0.01$). Following exercise βG was altered relative to pre-exercise ($P<0.05$, ANOVA). Highest activity was measured on day 5, although this was not significantly different from pre-exercise levels. No changes were detected in serum TAC, or the concentrations of MDA in plasma, or uric acid in serum.

*Muscle*

Following exercise G6PDH was elevated ($P<0.01$, ANOVA) to 330±151% of baseline on day 4 (NS) and 438±65% on day 7 ($P<0.05$, Newman-Keuls test). βG was elevated ($P<0.001$, ANOVA) to 171±16% of baseline on day 4 (NS) and 340±89% on day 7 ($P<0.05$, Newman-Keuls test). Total sulphydryls were altered from baseline ($P<0.05$, ANOVA), to 97±10% of the baseline concentration on day 4 (NS) and 175±35% on day 7 (NS). The TAC of the aqueous muscle extract was elevated ($P<0.01$, ANOVA) to 105±8% of baseline on day 4 (NS) and

146±17% on day 7. The TAC of the 'bound' muscle extract was elevated ($P<0.001$, ANOVA) to 202±51% of baseline on day 4 ($P<0.05$, Newman-Keuls test) and 258±54% on day 7 ($P<0.01$, Newman-Keuls test). Thus muscle bound TAC appeared to show an progressive increase following exercise, being significantly elevated on day 4 and showing further elevation on day 7. In contrast aqueous antioxidants were only elevated 4 days or more after exercise. No changes were observed in the muscle MDA concentration.

*Histology*

Baseline samples prepared for LM appeared normal, with tightly packed polygonal fibres, arranged into well defined fasicles. Nuclei were sparsely distributed in the *perimysium* and *endomysium*, with no central nuclei within fibres (Fig. 5.1). Although the exercise protocol produced evidence of muscle damage in all subjects the biochemical responses were highly variable between subjects. Varying degrees of inflammation were observed between subjects on day 4 after exercise. Some biopsies showed fibres with central nuclei (Fig. 5.2a), although most fibres maintained their normal appearance. Increased numbers of nuclei were also observed in the *endomysium* at this time (Fig. 5.2b). By day 7 after exercise cellular infiltration was more extensive and in some subjects resembled muscle myopathy, with an apparent loss of fibres (Fig. 5.3a). Many fibres appeared normal but were surrounded by nuclei (Fig. 5.3b), while other fibres were necrotic (Fig. 5.3c).

**Table 5.1 Serum TAC, uric acid concentration and KE soreness prior to and following eccentric exercise.**

| Parameter | Time (days) Pre 5 | Pre 3 | Pre Ex | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum TAC ($\mu$mol.Trolox Eq.l$^{-1}$) | 477 (31) | 501 (38) | 439 (38) | - | - | 502 (42) | 458 (34) | 485 (24) | 484 (30) | 487 (38) | - | - | 473 (45) | - | 487 (36) |
| Serum uric acid concentration ($\mu$mol.l$^{-1}$) | 271 (16) | 284 (19) | 282 (19) | - | - | 285 (19) | 275 (14) | 271 (13) | 275 (14) | 265 (21) | - | - | 280 (15) | - | 291 (18) |
| Muscle soreness (Arbitrary Units) | - | - | 6.0 (0) | 23 (14-33) | 36* (27-43) | 39* (24-48) | 27* (16-36) | 18* (13-24) | 12* (8-18) | 8.0 (6-13) | 6.0 (0) | 6.0 (0) | - | - | - |

*$P$<0.05, relative to pre-exercise.

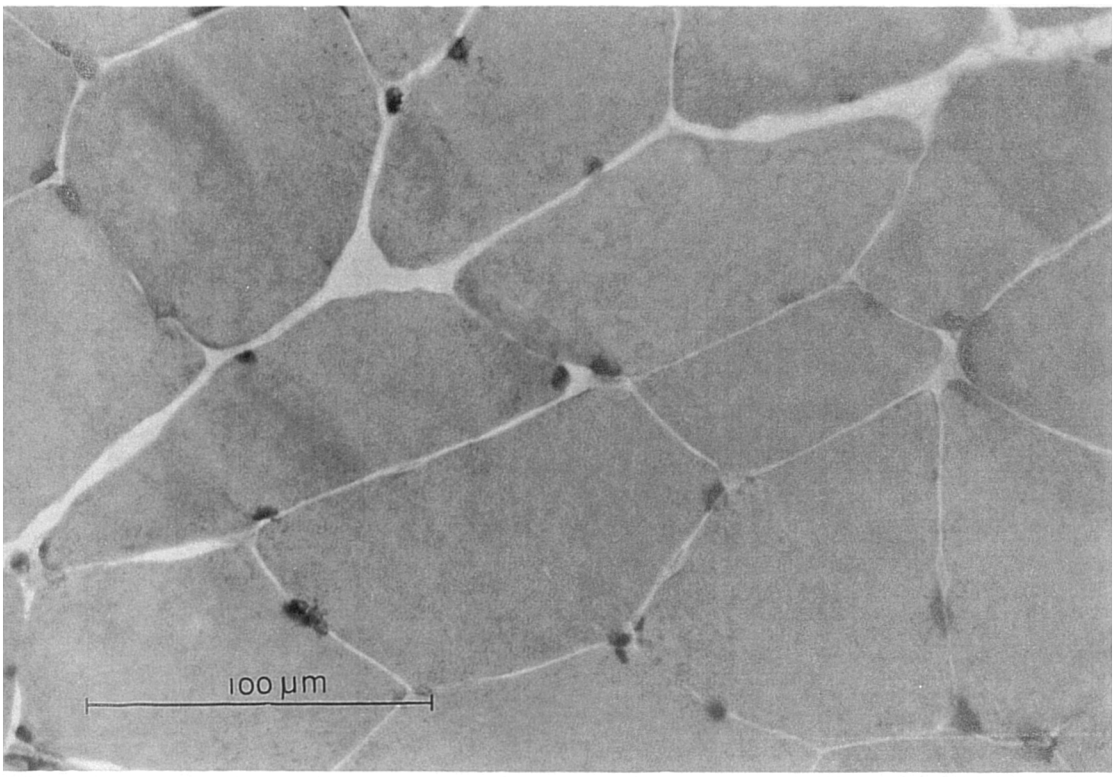


**Fig. 5.1 Representative light micrograph five days prior to exercise, showing polygonal fibres without cellular infiltration.**

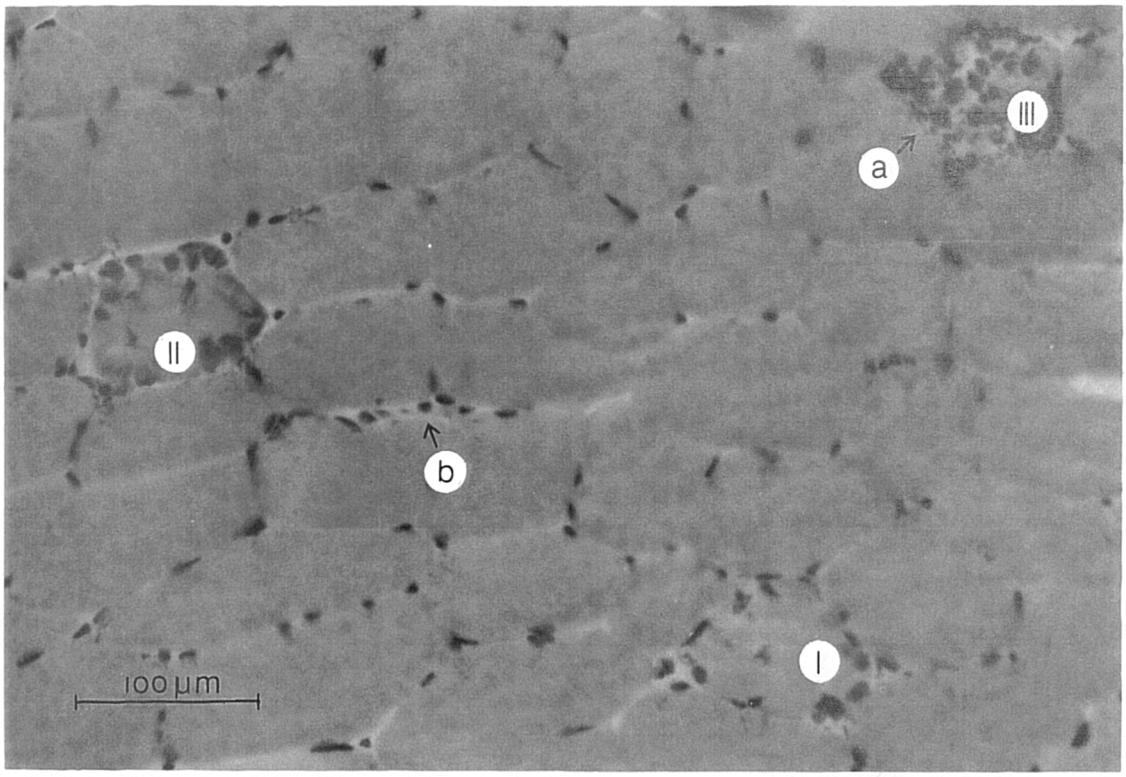


**Fig. 5.2 Representative light micrograph four days after exercise, showing increasing degrees of cellular infiltration in fibres I, II and III.**

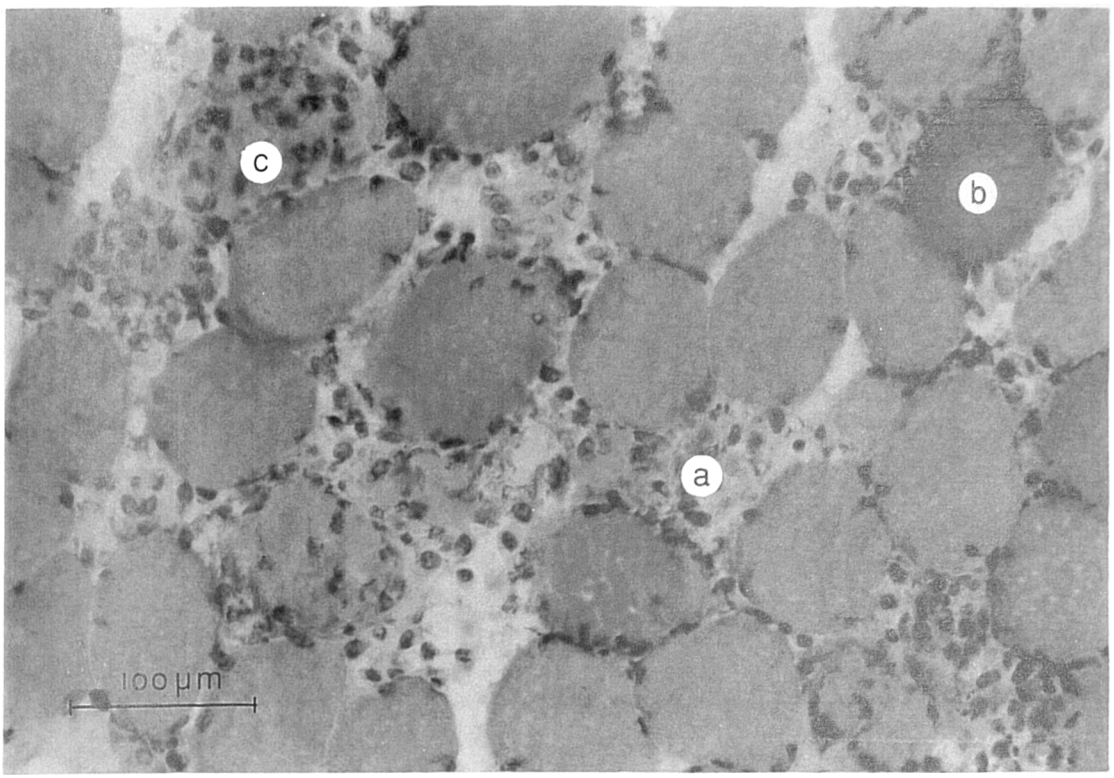


**Fig. 5.3 Light micrograph seven days after exercise, showi ıg extensive cellular infiltration.**

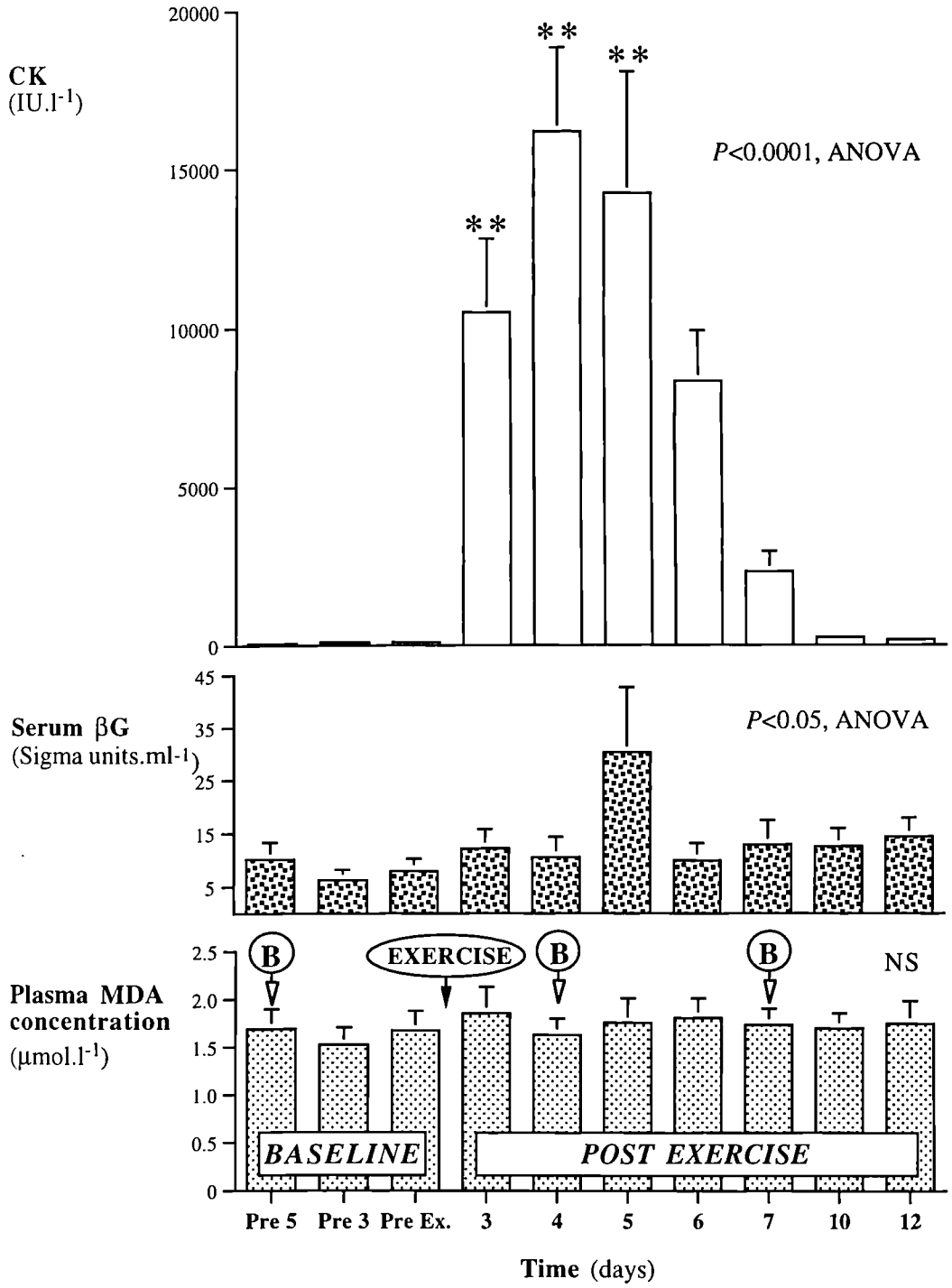


**Fig. 5.4 Changes in the serum activities of β-Glucuronidase, creatine kinase and plasma MDA concentration, in response to muscle biopsies (B), and eccentric exercise.** ** $P<0.01$, relative to pre-exercise.

**Table 5.2 Changes in muscle inflammatory indices and antioxidants in response to eccentric exercise.**

| Parameter | Baseline | Day 4 | Day 7 | Statistical changes |
|---|---|---|---|---|
| β-Glucurondase<br>(log pico gram phenolphthalein formed. hour $^{1}$. gram muscle wet weight$^{-1}$) | 4.94<br>(0.04) | 5.17<br>(0.06) | 5.42 **<br>(0.11) | $P<0.001$, ANOVA |
| G6PDH<br>(log pico mol $NADP^{+}$ reduced $min^{-1}$ . gram wet weight muscle $^{-1}$) | 4.72<br>(0.15) | 5.07<br>(0.06) | 5.36 *<br>(0.19) | $P<0.01$, ANOVA |
| Total sulphydryls<br>(log pico mol. gram wet weight muscle$^{-1}$) | 2.47<br>(0.03) | 2.45<br>(0.03) | 2.66<br>(0.05) | $P<0.05$, ANOVA |
| Aqueous TAC<br>(log pico mol Trolox Eq.gram wet weight muscle$^{-1}$) | 6.11<br>(0.05) | 6.12<br>(0.03) | 6.26 *<br>(0.02) | $P<0.01$, ANOVA |
| Bound extract TAC<br>(log picomol Trolox Eq. gram dry weight muscle$^{-1}$) | 7.38<br>(0.06) | 7.55 *<br>(0.06) | 7.70 **<br>(0.06) | $P<0.001$, ANOVA |
| Malondialdehyde<br>(Log pico mol. gram wet weight muscle$^{-1}$) | 5.07<br>(0.06) | 4.72<br>(0.15) | 5.36<br>(0.19) | NS |

*$P<0.05$, **$P<0.01$ relative to baseline.

## Discussion

An experimental muscle damage model was used to investigate the possibility of free radical induced muscle injury, resulting from inflammation. The protocol employed had the advantage of damaging a specific muscle group, thereby allowing indirect damage indices to be related to changes in the damaged musculature. Classic responses in muscle damage indices were observed following exercise, including impaired muscle contractility, with delayed elevations in MS and CK (Fig. 5.4). In the exercised muscle a progressive increase in cellular infiltration was observed. These changes are well documented following muscle damaging exercise in humans (Friden *et al.* 1983, Schwane *et al.* 1983, Jones *et al.* 1986).

Eccentric exercise resulted in a substantial rise in CK, which was maximal 4 days after exercise. Exercise also produced elevations in serum βG. As these serum indices of muscle damage were not influenced by the baseline muscle biopsy, it would appear this procedure did not produce significant muscle injury. Therefore, the rise in CK and βG following the eccentric muscle actions were probably a consequence of exercise induced muscle damage.

Techniques for injury quantification using light microscopy, which use the number and location of nuclei to classify fibres as 'damaged' (e.g. Aharata & Engel 1984) have many limitations, which have been discussed previously by Faulkner *et al.* (1993) and Komulainen & Vihko (1994). Faulkner *et al.* (1993) proposed the decline in maximum force generation is the most valid measure of muscle damage. The magnitude of change in MVC and 20:100 force ratio in this study suggest exercise resulted in muscle damage, although fatigue may also have contributed to the force deficits following exercise. In an attempt to minimise ultrastructural damage artifacts no contractile measurements were made in the days following exercise. It has been proposed the generation of high muscle forces might produce further disruption to fibres which were damaged, or undergoing repair (Friden *et al.* 1983).

In this study muscle βG was determined in an attempt to overcome limitations in the quantification of muscle damage using histological and functional measures. The activity of the lysosomal

enzyme βG closely reflects exercise induced muscle damage in rodents (Vihko & Salminen 1986, Komulainen & Vihko 1994), being significantly correlated with the histopathologic state of the muscle (Salminen & Kihlstrom 1985). Macrophages present in the connective tissue of normal muscle, appear to be the main source of βG, although some activity is also present within the muscle fibres (Vihko *et al.* 1978). Increased βG in damaged mouse muscle originates both from surviving muscle fibres (without cellular infiltrates) and phagocytes (Vihko *et al.* 1978). Immunohistochemical studies of exercise damaged muscle have shown β–Glucuronidase to localise between myofibrils and in connective tissue (Vihko *et al.* 1978) and such structures are known to be damaged by exercise (Friden *et al.* 1983, Takala *et al.* 1986, Stauber *et al.* 1990). This may make muscle βG a more sensitive marker of muscle damage than histological analysis using light microscopy. In the present study elevations in muscle βG suggest KE damage increased between days 4 and 7 after exercise. Increased activity of G6PDH has been used as a marker of cellular infiltration in exercise damaged rodent muscle (Tullson & Armstrong *et al.* 1981, Warren *et al.* 1992), as macrophages demonstrate high activity of this enzyme relative to muscle tissue. In the present study the changes G6PDH suggest there was increased cellular infiltration between days 4 and 7 after exercise.

Biochemical evidence of inflammation (Table 5.2) was verified by histological analysis, which showed increased cellular infiltration between days 4 and 7 after exercise (Figs 5.1, 5.2 and 5.3). The magnitude of cellular infiltration was highly variable between subjects, as in previous studies (Jones *et al.* 1986). In one subject extensive cellular infiltration was observed, with aggregations of nuclei in the *endomysium* and possibly *perimysium* (Fig. 5.3). These observations are similar to previous reports in humans (Round *et al.* 1987, Stauber *et al.* 1990) and may reflect exercise induced connective tissue damage (Tullson & Armstrong 1981). The time scale of these changes are consistent with previous reports of exercise myopathy in humans (Jones *et al.* 1986, Round *et al.* 1987) but differ from reports in mice and rats. Following damaging exercise in rodents evidence of infiltration has been reported to commence within 12 hours of exercise (Komulainen & Vihko 1994); with maximal cellular infiltration between 1 and 3 days after exercise (Salminen & Kihlstrom 1985). Previous reports suggest the peak in cellular infiltration, CK and force loss may coincide in

rodents. In this study, as in previous studies on humans (e.g. Jones *et al.* 1986) peak CK preceded maximum cellular infiltration.

The activity of β–Glucuronidase and G6PDH appeared to reflect the increased numbers of nuclei in muscle biopsy specimens. The release of acid hydrolases such as β–Glucuronidase and cellular infiltration are characteristic of inflammation. These events suggest neutrophils and macrophages were activated, which is also associated with the production of oxygen radicals (Henson & Johnston 1987, Weiss 1989). A variety of inflammatory disease states have been associated with reduced serum TAC (Whitehead *et al.* 1992, Mulholland & Strain 1991), while rhabdomyolysis has been associated with hyperuricaemia (Knochel 1982). Despite evidence for inflammation and rhabdomyolysis no changes were observed in serum TAC, or uric acid in this study (Table 5.1).

In biopsy specimens TAC in aqueous and bound muscle extracts were elevated (Table 5.2), as were aqueous sulphydryls. However using the techniques employed no distinction can be made between antioxidants in muscle fibres and cellular infiltrates. Inflammatory cells have been reported to elevate cysteine and reduce tocopherol levels in human neoplastic breast tissue (Langemann *et al.* 1989). Histological analysis suggested many biopsy specimens did not show substantial inflammation. It is possible therefore that the relatively small number of infiltrating cells may not have produced the increases in aqueous and bound antioxidants directly. Therefore, the elevations in aqueous TAC and sulphydryls may represent changes within the muscle fibres. Several similarities exist between the changes in aqueous antioxidants in this study and previous experiments reporting responses to oxidative stress (Maulik *et al.* 1995, Wu *et al.* 1996). Such investigations may help to elucidate possible mechanisms by which aqueous antioxidants were elevated in the present study.

Rajguru *et al.* (1994) have previously observed an increase in the sulphydryl content of rodent muscle 30 minutes after swimming exercise. The authors suggested this may have reflected increased cysteine or reduced glutathione in skeletal muscle. Similarly Maulik *et al.* (1995) reported endotoxin induced oxidative stress increased ascorbate and thiol components of the antioxidant reserve in rat myocardium. Maulik *et al.* (1995) proposed these responses could reduce myocardial oxidative injury. This proposal is consistent with the

observations of Wu *et al.* (1996), who found increased cellular glutathione maintained the viability of human astrocytoma cells during oxidative stress. The elevation in aqueous antioxidants in the present study may provide evidence that similar changes occur in human muscle following damaging exercise. Furthermore such responses might reduce undesired oxidation by infiltrating cells and/or could aid in tissue repair (Allen 1991, Rajguru *et al.* 1994).

Changes in bound and aqueous TAC (Table 5.2) suggest elevations in bound antioxidants were not simply a reflection of changes in the aqueous environment. In addition to its role as an aqueous antioxidant glutathione is also concentrated in lipid membranes (Mbemba *et al.* 1984). The rise in bound TAC in the present study may be consistent with an increase in membrane bound antioxidants. If such a response occurred in the sarcolemma it might increase resistance to oxidative cellular injury, and/or facilitate biosynthesis in cells which sustained sublethal injury.

No changes in the MDA concentration in muscle and plasma were detected in response to exercise. Initially this result is surprising when considering the magnitude of inflammation present in the muscle, which would typically be associated with an increase in free radical stress (Weiss 1989). However, free radical damage may have been minimised or even prevented by the rise in muscle antioxidant status. Inflammation without evidence of peroxidation in muscle has been reported in exercise myopathy in mice (Salminen & Vihko 1983) and muscular dystrophy in both mice (Asayama *et al.* 1989) and humans (Burr *et al.* 1987). To the authors knowledge however this is the first study to evaluate inflammatory and peroxidative indices in human exercise myopathy.

It is apparent from this and previous studies, that the peak in CK and isometric force loss occur before the peak in cellular infiltration in humans. Despite evidence of muscle damage and inflammation in this study, muscle antioxidant status was not compromised, and MDA was not altered in muscle or plasma. Therefore, this investigation provides no evidence that the prolonged and delayed release of creatine kinase is caused by compromised antioxidant protection and subsequent lipid peroxidation.

# CHAPTER 6

## Functional and biochemical changes in indices of muscle damage, lipid peroxidation and lysosomal enzyme release, following eccentric exercise in humans.

## Summary

The plasma concentration of malondialdehyde (MDA), and indices of muscle damage were used to evaluate if lipid peroxidation might contribute to indices of delayed muscle damage following eccentric exercise. Eight subjects (6 female, 2 male) each performed 50 maximal voluntary eccentric muscle actions on an isokinetic dynamometer, using the knee extensors (KE) of a single leg. Maximum voluntary contractile force (MVC) and the 20:100 force ratio were determined immediately before and following exercise, and then again on days 1, 2, 3, 7, and 9. Venous blood was collected before and on days 1, 2, 3, 7 and 9 following exercise. Serum creatine kinase activity (CK) and serum β-Glucuronidase activity (βG) were used as indices of sarcolemmal damage and lysosomal enzyme release respectively. Muscle soreness (MS) was evaluated before and on each day following exercise. Parametric data were analysed using analysis of variance (ANOVA) and Newman-Keuls tests. Exercise produced decrements in both MVC and the 20:100 force ratio ($P<0.0001$, ANOVA), with a peak in MS 2 days after exercise. CK was elevated ($P<0.001$, ANOVA) with highest recorded activity 3 days after exercise ($P<0.01$). Changes in MDA were also observed ($P<0.05$, ANOVA), with the highest plasma concentration observed 2 days after exercise ($P<0.05$). Serum βG did not show significant changes following exercise. The elevation in MDA before peak CK, MS and force loss suggest peroxidation may contribute to these indices of delayed muscle damage.

## Introduction

High force eccentric muscle actions typically result in a peak in serum creatine kinase activity (CK) 2 to 4 days after exercise, although the mechanisms underlying this delayed rise in CK have not been elucidated. Meydani *et al.* (1993) have reported a biphasic rise in urinary thiobarbituric acid adducts (which include malondialdehyde), following downhill running at 75% $VO_2$ max. In subjects less than 30 years old an initial peak was observed 2 days after exercise, with a second and larger peak 12 days after the run. It is unfortunate Meydani *et al.* (1993) did not assess any muscle damage indices, as such measures may have helped to elucidate the relationship between peroxidation and the discharge of myocellular constituents.

One possibility is that peroxidation of the sarcolemmal membrane results in enzyme release, which has been demonstrated following ischaemia (Gauduel *et al.* 1989, Lazzarino *et al.* 1994). This study was conducted to determine if lipid peroxidation was associated with initial elevations in CK, muscle soreness and impairments in contractile function, following eccentric exercise.

## Methods

*Subjects*

Eight subjects (6 female and 2 male), who had not been involved in weight training activities for the previous 6 months volunteered for the study. Their mean±SE age and mass were 22±1 years and 69±2.6 kg. Experimental procedures were approved by Wolverhampton University ethics committee and all subjects gave written informed consent prior to their participation in the study.

*Isometric force measurement*

Knee extensor force was measured in a seated position at a knee flexion angle of 1.57 rad., using a strain gauge system described previously (Section 2.2). Maximum voluntary contractile force (MVC) with PES and the 20:100 force ratio were determined using techniques described in sections 2.2 and 2.3.

*Exercise*

Each subject performed 50 maximal voluntary eccentric muscle actions on the isokinetic dynamometer described in section 2.1, using the knee extensors of a randomly selected leg. Exercise was performed in a prone position using a range of motion from 2.97 to 1.21 rads (170° to 70°). A preload was used such that each subject produced maximal knee extensor force prior to movement of the dynamometer lever arm. This allowed isometric force to rise before the eccentric action, which was performed at an angular velocity of 1.05 $rad.s^{-1}$. Each repetition was separated by a 10 second rest period, during which the leg was returned to the start position by the experimenter at 1.05 $rad.s^{-1}$.

*Muscle Soreness (MS)*

Soreness was assessed before exercise and on each subsequent day at 8 muscle regions (6 extensor, 2 flexor) depicted on a questionnaire (Appendix 1). Soreness evaluation was performed *via* palpation of 6 muscle sites as previously described (section 2.4). Each subjects soreness values (for the 6 KE muscle sites) were summed each day, and the total used as a criterion score

*Blood collection and analysis*

Blood samples were collected as described in section 2.5 before exercise and on days 1, 2, 3, 7 and 9 thereafter.

*Creatine kinase activity*

Serum creatine kinase activity (CK) was determined using the protocol described in section 2.6.

*β–Glucuronidase activity* (βG)

Serum β–Glucuronidase activity was determined using the protocol described in section 2.8.

*Malondialdehyde* (MDA)

The plasma concentration of malondialdehyde was determined using the protocol described in section 2.12.

*Statistics*

Statistics were performed using techniques described in section 2.13.

## Results

Exercise resulted in a changes in MVC ($P<0.0001$, ANOVA) with a significant decline on day 1 ($P<0.05$, Newman-Keuls test) and lowest force recorded 3 days after exercise ($P<0.01$, Newman-Keuls test). Changes in the 20:100 were also observed after exercise ($P<0.0001$, ANOVA), with a significant decline post exercise ($P<0.05$, Newman-Keuls test). Changes in muscle contractile function are shown in Fig. 6.1.

After exercise, CK rose from $42\pm16$ IU.l$^{-1}$ pre-exercise, to a maximum recorded value of $2{,}816\pm1{,}465$ IU.l$^{-1}$, 3 days after exercise. Following logarithmic transformation, pre-exercise CK was altered ($P<0.001$, ANOVA) with elevations on days 3 and 7 after exercise ($P<0.01$, Newman-Keuls test). Changes in MDA were observed following exercise ($P<0.05$, ANOVA) from $1.30\pm0.09$ μmol.l$^{-1}$ pre-exercise to a maximum recorded value of 1.47 μmol.l$^{-1}$, 2 days after exercise ($P<0.05$, Newman-Keuls test). Serum β–Glucuronidase activity rose from $20{,}300\pm700$ Sigma units.l$^{-1}$ pre-exercise, to a maximum recorded value of $28{,}200\pm6{,}200$ Sigma units.l$^{-1}$ 9 days after exercise, although this change was not statistically significant. Biochemical responses are shown in Fig. 6.2. Muscle soreness was reported in the KE following exercise on days 1 to 6 after exercise ($P<0.05$, Wilcoxon test), with peak soreness on day 2 (Fig. 6.3).

Two days after exercise a significant correlation was observed between CK and MS ($r=0.983$, $P<0.0001$). LFF and MDA were also significantly correlated two days after exercise ($r=-0.886$, $P<0.05$, Pearson correlation). At the same time point MDA did not correlate significantly with MVC, CK or MS, giving $r$ values of -0.708, 0.690 and 0.555 respectively.

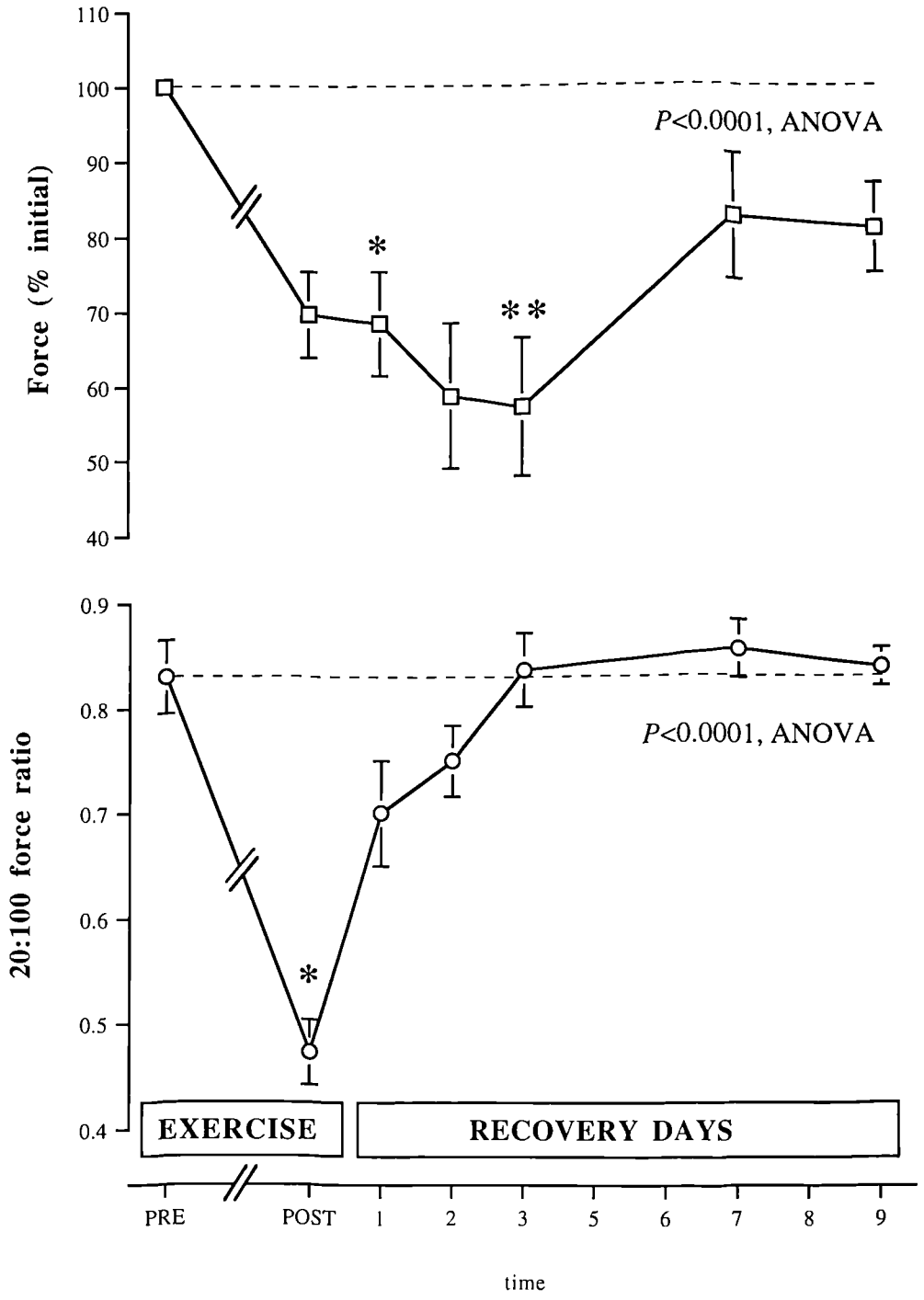


**Fig 6.1 Changes in MVC and the 20:100 force ratio following exercise.**

* $P<0.05$, ** $P<0.01$, relative to baseline.

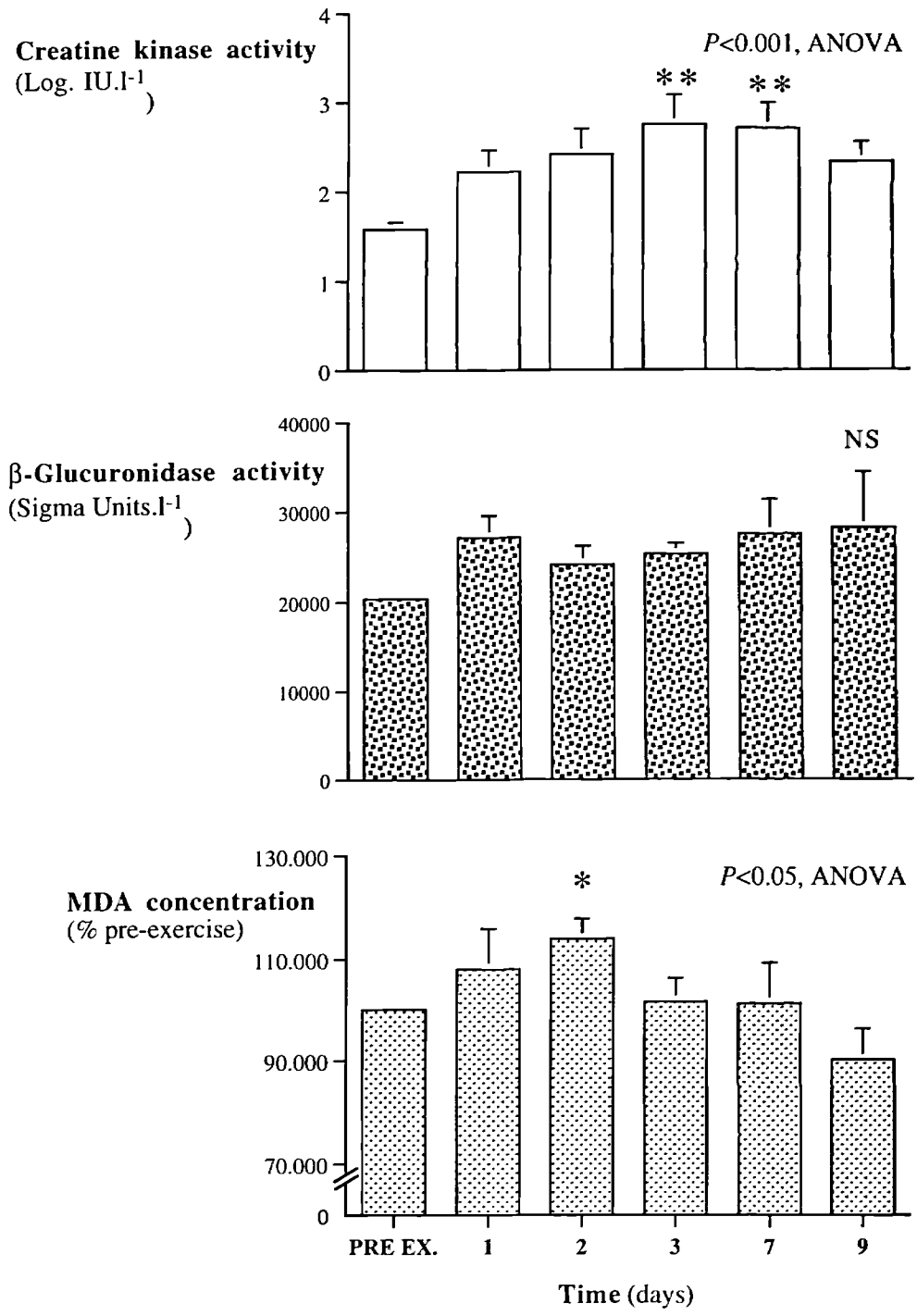


**Fig. 6.2 Changes in serum activities of β-Glucuronidase, Creatine kinase and plasma malondialdehyde concentration following exercse.**

* $P<0.05$, ** $P<0.01$, relative to pre-exercise.

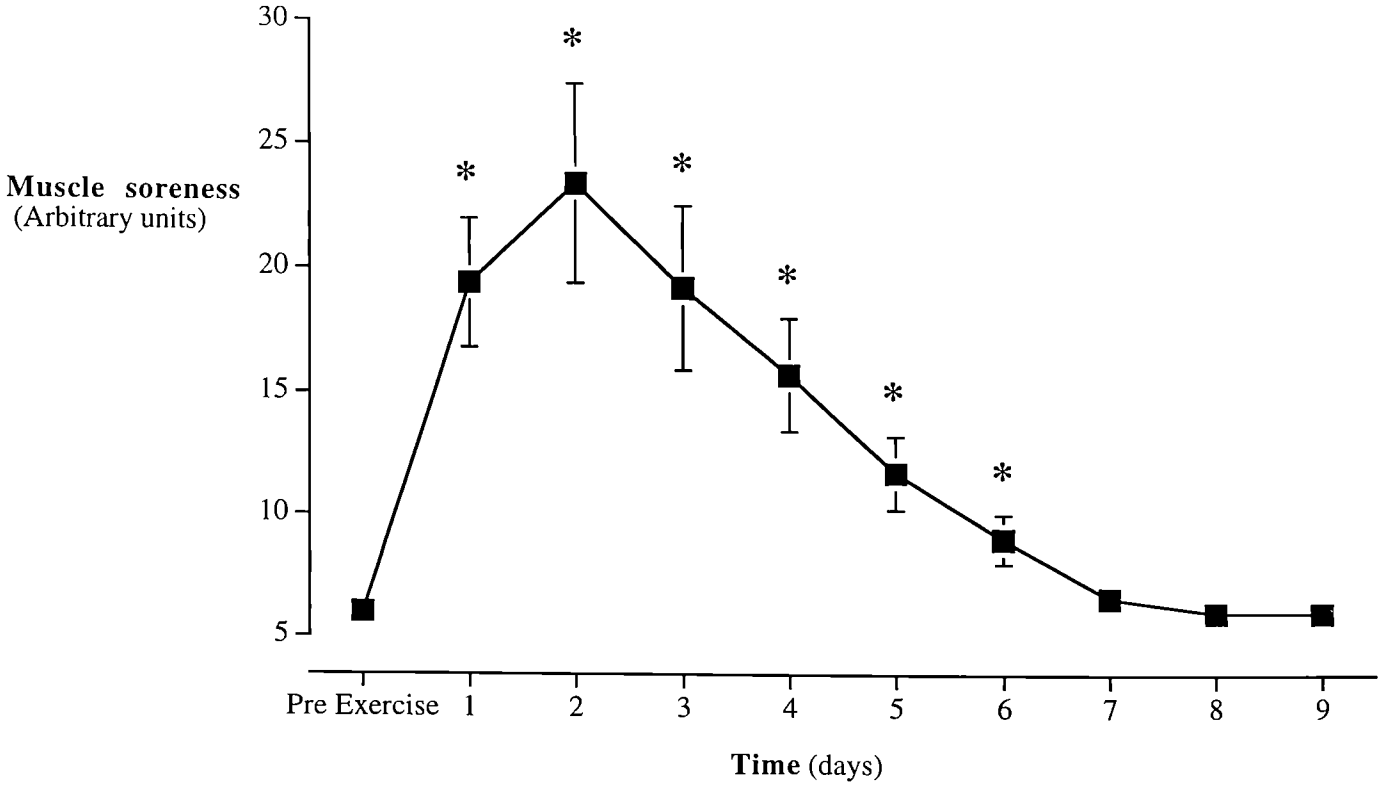


**Fig. 6.3 Changes in muscle soreness following 50 maximal voluntary eccentric muscle actions.**

Soreness values are means for 6 sites on the knee extensors. This gives a value of 6 for no soreness, with a maximum soreness of 60. * $P<0.05$, relative to pre-exercise.

## Discussion

Subsequent to exercise, delayed elevations in CK and MS were observed. These changes were consistent with previous investigations using high force eccentric muscle actions reported in the literature (Clarkson 1992) and in this thesis (Chapters 3, 4 and 5). The delayed decline in maximum isometric force (Fig. 6.1) is an unusual phenomenon in humans, though it has previously been reported in mouse muscle following lengthening contractions (Zerba *et al.* 1990). These authors found SOD administration attenuated the decline in force 3 days after exercise. To explain this effect Warren *et al.* (1992) and Witt *et al.* (1992) speculated that infiltrating phagocytes may have resulted in free radical mediated tissue injury. However indices of muscle antioxidant status and free radical damage were not evaluated by Zerba *et al.* (1990). In a separate study Duarte *et al.* (1994) reported a reduction in the glutathione concentration of mouse soleus muscle, 3 days after exhaustive running. Thus in rodent muscle, the time course of immunological (Komulainen & Vihko 1994), biochemical (Duarte *et al.* 1994) and functional changes (Zerba *et al.* 1990), may be consistent with inflammation mediated free radical damage.

The rise in MDA following exercise suggests an imbalance between free radical production and antioxidant defenses in favour of the former. This situation only appeared to arise in the first two days after exercise, as MDA was very similar to baseline levels thereafter (Fig. 6.2). These findings are akin to those of Meydani *et al.* (1993), although the present investigation may not have followed a sufficiently long time course to detect a second peak in MDA. It has been reported damaging eccentric exercise performed with the arm biceps did not elevate serum MDA (Saxton *et al.* 1994). The experimental model used by these investigators may have damaged a smaller mass of muscle tissue than the model used in the present investigation. Therefore although elevations in MDA may have occurred in muscle, these changes may not have been detected in serum as a consequence of dilution effects.

Two days after exercise the plasma MDA concentration correlated significantly with LFF. Several studies have implicated free radicals in LFF (Reid *et al.* 1992) and decrements in the generation of

peak muscle force (Nashawati *et al.* 1993). Jakeman & Maxwell (1993) proposed LFF might arise from oxidative damage to the sarcoplasmic reticulum (SR). It should be recognised that compromised functioning of structures other than SR could also result in LFF and these changes could be indirect consequences of oxidative damage. There were also non significant trends to suggest lipid peroxidation was associated with elevations in CK, MS and the loss of force at this time. The enzymatic changes in CK and serum βG activity were consistent with the loss of sarcolemmal and lysosomal membrane integrity, which can result from membrane peroxidation (Lazzarino *et al.* 1994, Fong *et al.* 1973). The associated production of compounds such as arachidonic acid and prostaglandins (Hong *et al.* 1989), can induce sensations of soreness (Hayward *et al.* 1991). Thus, free radical mediated injury may also have contributed to delayed elevations in CK, muscle soreness and the loss of force following exercise.

There are several mechanisms by which exercise can increase free radical production. Witt *et al.* (1992) proposed exercise may produce ischaemic reperfusion injury of muscle tissue. This is an unlikely explanation for the rise in CK and MDA in the present study for several reasons. Firstly moderate circulatory demands imposed by high force eccentric exercise are also unlikely to produce ischaemia of sufficient severity or duration to produce muscle injury. It has also been demonstrated human striated muscle is extremely resistant to ischaemic reperfusion injury (Mair *et al.* 1995). Witt *et al.* (1992) also proposed the production of reactive oxygen species by phagocytes could also increase oxidative stress in exercise damaged muscle. From the techniques employed in the present study it is impossible to determine if the exercise bout resulted in muscle inflammation. If such events did occur, previous studies in humans (Jones *et al.* 1986, Round *et al.* 1987, Chapter 5) would suggest inflammation occurred after the initial release of CK, and peak in MDA. As muscle inflammation does not appear toresult in increased peroxidation (Chapter 5), this appears an unlikely explanation for the peak in MDA. Overproduction of free radicals by mitochondria is also recognised as a potential cause of free radical mediated injury during exercise (Sjodin *et al.* 1990, Witt *et al.* 1992). Surprisingly, relatively few studies have acknowledged mitochondria as a source of increased free radical stress following exercise, except in relation to ischaemic reperfusion injury.

A number of mitochondrial abnormalities have observed following ischaemic reperfusion injury of striated muscle which have many commonalities with the changes associated with damaging exercise. Mitochondrial swelling (Friden *et al.* 1983), intramitochondrial crystals (Hikida *et al.* 1983, Komulainen & Vihko 1994) and a reduction in the respiratory control ratio (Davies *et al.* 1982) have been reported in exercise damaged muscle. Such changes provide indirect evidence of mitochondrial injury, which following exercise might arise from the mitochondrial membrane peroxidation (Bus & Gibson 1979) or elevated mitochondrial calcium (Halestrap *et al.* 1993). These events are closely associated with aberrations in mitochondrial metabolism and excessive production of reactive oxygen species (Gauduel *et al.* 1989, Beal 1996). It is possible increased radical formation by the mitochondria of damaged fibres might be responsible for the rise in MDA 2 days after exercise.

The results of the present study suggest lipid peroxidation may have contributed to delayed MS, CK and possibly force loss. The present investigation and previous studies (Meydani *et al.* 1993), indicate antioxidant supplements may be justified to reduce oxidative damage following eccentric exercise.

# CHAPTER 7

## Changes in serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half marathon run.

Aspects of this study were presented at the Symposium on distance running, British Association of Sports Sciences Annual Conference 1996, Lilleshall, UK.

This study is in press, in abstract form (R.B. Child, D.M. Wilkinson, J.L. Fallowfield, A.E. Donnelly 1996 Changes in serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half marathon run. Journal of Sports Sciences, 1997).

I gratefully acknowledge the work of D.M. Wilkinson and J.L. Fallowfield in designing and conducting the experiment. I would also like to thank them for their collection of physiological data during exercise and evaluation of lactate and cortisol concentrations.

**Summary**

Indices of antioxidant status, membrane permeability, and lipid peroxidation were investigated in venous blood immediately before and after a simulated half marathon run. These were the ability of serum to quench free radicals (total antioxidant capacity, TAC), the serum uric acid concentration (UA), serum creatine kinase activity (CK) and serum β-Glucuronidase activity (βG). The plasma concentration of malondialdehyde (MDA) was used as a marker of lipid peroxidation. Data were analysed with paired *t*-tests. Following a standardised warm-up, 17 trained male runners (means±SD, age 31±4 years, peak $VO_2$ 63.2±4.8 $ml.kg^{-1}.min^{-1}$) each completed a self paced half marathon run, on a motorised treadmill. Average exercise intensity was 77.1±1.0% peak $VO_2$, with a performance time of 87.1±7.0 minutes. Following exercise elevations were observed in plasma MDA from 1.48±0.39 $\mu mol.l^{-1}$ to 1.65±0.32 $mmol.l^{-1}$ ($P<0.05$), serum TAC from 475±84 to 564±113 μmol Trolox $Eq.l^{-1}$ ($P<0.0001$), UA from 268±45 to 312±51 $\mu mol.l^{-1}$ ($P<0.001$), serum cortisol concentration from 339±95 to 557±157 $nmol.l^{-1}$ ($P<0.01$), CK from 98±67 to 133±89 $IU.l^{-1}$ ($P<0.0001$), βG from 15.39±5.34 to 17.05±5.7 Sigma $Units.ml^{-1}$ ($P<0.001$). The rise in serum TAC, did not prevent exercise induced lipid peroxidation and muscle damage as both plasma MDA and CK were elevated following exercise. This may indicate inadequacies in the antioxidant defence system during the half marathon run.

## Introduction

During intense exercise, whole body oxygen uptake can increase 10 fold above resting levels (Mitchell *et al.* 1958), however in active muscle fibres oxygen consumption may rise 200 fold (Keul *et al.* 1972). *In vitro* experiments suggest 2 to 5% of total electron flux through the cytochrome chain results in superoxide radical formation (Boveris & Chance 1973, Pryor 1986). The presence of such mitochondrial metabolic leaks may result in a substantial increase in radical oxidative stress (ROS) during exercise (Davies *et al.* 1982, Jenkins 1993). The production of oxygen radicals can result in membrane peroxidation and malondialdehyde (MDA) formation (Kramer *et al.* 1984).

*In vivo*, free radical damage is minimised by a complex antioxidant defense system, part of which involves the interception of free radicals with sacrificial antioxidants. In plasma, ascorbate provides substantial protection against free radical damage (Frei *et al.* 1989), although the high concentration of uric acid also makes this an effective antioxidant *in vivo* (Ames *et al.* 1981, Miller *et al.* 1993 Whitehead *et al.* 1992). When assessing antioxidants individually, it is necessary to determine the concentration of a spectrum of compounds to provide an index of total free radical protection. Developments in assay techniques now allow the total free radical quenching capacity of complex antioxidant solutions to be quantified. The method of Whitehead *et al.* (1992) has proved effective in detecting reduced serum total antioxidant capacity (TAC) in disease states associated with increased free radical stress (Whitehead *et al.* 1992).

Elevations in circulating antioxidants can occur during aerobic exercise (Gleeson *et al.* 1987, Duthie *et al.* 1990, Maxwell *et al.* 1993, Viguie *et al.* 1993), which may increase serum TAC (Maxwell *et al.* 1993). While some studies report increased MDA following exercise (Lovlin *et al.* 1987, Kanter *et al.* 1988, Kanter *et al.* 1993), others provide no evidence for exercise induced free radical damage (Duthie *et al.* 1990, Sahlin *et al.* 1991, Maxwell *et al.* 1993, Viguie *et al.* 1993). These conflicts may be attributable to methodological differences, especially in relation to exercise duration and intensity, which contribute to the occurrence of free radical damage (Lovlin *et al.* 1987). Training status may also influence susceptibility to oxidative injury as

regular exercise appears to enhance some components of the antioxidant system (Higuchi et al. 1985, Robertson *et al.* 1991, Kretzschmar *et al.* 1991, Criswell *et al.* 1993).

Many athletes are engaged in high intensity endurance exercise training. To the authors knowledge the influence of this type of activity, on serum antioxidant capacity has not been reported previously. Therefore this study examined the effects of a simulated half marathon run on serum TAC, plasma MDA, and indices of sarcolemmal and lysosomal membrane permeability in trained runners.

## Methods

*Subjects*

Seventeen trained runners volunteered for the study. Their mean±SD age, mass, weekly training distance, and peak $VO_2$ were 31±4 years, 71.0±5.0 kg, 47±19 km and 63.2±4.8 $ml.kg^{-1}.min^{-1}$ respectively. Experimental procedures were approved by Chichester Institute ethics committee, and all subjects gave written informed consent prior to their participation in the study.

*Experimental protocol*

To determine average weekly training distance, all subjects recorded their training mileage for four weeks, prior to the start of the simulated half-marathon run. Subjects refrained from exercise for 36 hours before each laboratory testing session, and performed only light training in the 2 days before exercise tests.

Within a two week period before the half marathon, each subject completed a graded exercise test to determine individual speed-oxygen uptake regression equations. The test involved completion of four to six 4-minute stages, on a motorised treadmill (Quinton Q65, Quinton Instruments, Seattle, USA). Expired gas was collected in the final minute of each stage using standard Douglas bag techniques. A fingertip blood sample was collected at the end of each exercise stage to determine the capillary blood lactate concentration (Bla). Following blood collection, running velocity was increased progressively by 1.5 $km.h^{-1}$ and the test was terminated when the (BLa) exceeded 4 $mmol.l^{-1}$.

Following a 30 minute recovery period, subjects performed an incremental graded test to establish peak $VO_2$. Initial running velocity was set 2 $km.h^{-1}$ slower than the velocity which elicited a BLa of 4 $mmol.l^{-1}$ in the previous test. The gradient was increased by 1.5% every 90 s throughout the test. Expired gas was collected continuously from 5 minutes, to test termination. Interpolation of the speed-oxygen uptake regression were used to determine the running velocity required to elicit 50% and 70% of peak $VO_2$.

Subjects reported to the laboratory following a 3 hr fast and stood for 20 minutes prior to the half marathon to normalise for postural changes in blood volume. Immediately before exercise a 13 ml blood sample was drawn from an antecubital vein. Each subject then performed a controlled warm up on the treadmill, at pre-determined speeds corresponding to 50% peak $VO_2$ for 5 minutes and 70% peak $VO_2$ for 10 minutes. Thereafter, subjects were allowed to run self paced, and were encouraged to cover the remaining distance in the shortest possible time. This was achieved by enabling the subjects to adjust the velocity of the treadmill during the run. Capillary BLa was measured pre-exercise, during the warm up period at 5, 10, and 15 minutes, at 4, 8, 12, 16 and 20 kms, and then again immediately post-exercise. A further 13 ml of venous blood was drawn from an antecubital vein immediately post-exercise. Oxygen uptake was recorded during exercise, just prior to collection of capillary blood. To simulate race conditions subjects were allowed water *ad libitum* at 5, 10, 15, and 19 kms.

The volume of expired air was measured using a previously calibrated dry gas meter (Harvard Apparatus, Kent, UK). Gas fractions were determined using a paramagnetic oxygen analyser and infra red carbon dioxide analyser (Servomex 1400, Crowborough, UK), calibrated with nitrogen, two gravimetrically determined calibration gases (Linde Gases UK Ltd., London, UK) and ambient air. Prior to analysis all gases were saturated with water by passage through Nafion tubing (Omnifit Ltd, Cambridge, UK) immersed in distilled water.

*Biochemical analysis*

The whole blood lactate concentration of capillary blood samples was determined immediately following collection. This was performed using an automated analyser (Model 2300 Stat plus, Yellow Springs Inc., Ohio, USA).

In venous blood, haemoglobin concentrations were determined by the cyanmethaemoglobin method using a diagnostic kit (No. 124 729, Boehringer Mannheim, Mannheim, Germany). Haematocrit was determined in triplicate following microcentrifugation (Hawksley & Sons Ltd, Lancing, UK). The remaining venous blood was dispensed

into potassium EDTA tubes and was immediately centrifuged at 2,500*g* for 10 minutes, or plain tubes (to be centrifuged using the same protocol after clotting at room temperature for 1 hour). Plasma and serum were removed, and stored at -20°C until analysis. Measurements in venous blood were corrected for plasma volume changes using the method described by Dill & Costill (1974).

Serum antioxidant capacity was determined using the techniques described in section 2.11. The plasma MDA concentration was determined using techniques described in section 2.10.

Serum activities of creatine kinase and β-Glucuronidase were determined using the techniques described in section 2.6 and 2.8 respectively, with the serum uric acid concentration determined as described in section 2.9. The concentration of serum cortisol was determined using an automated ELISA system (Model ES300, Boehringer Mannheim) with a cortisol kit (Boehringer Mannheim, No. 1098 578).

Statistical analysis

All data are presented as means±SD. BLa data were analysed using a one-way repeated measures analysis of variance (ANOVA); all other data were analysed with paired *t*-tests and Pearson correlations.

## Results

The half marathon run was successfully completed by all subjects with a mean performance time of 87.1±7.0 minutes. The BLa rose during exercise ($P<0.0001$, ANOVA). During the self paced run average $VO_2$ was 3.4±0.1 $l.min^{-1}$ corresponding to an exercise intensity of 77.1±1.0 % peak $VO_2$. Data are presented in table 7.1.

Pre-exercise CK and weekly training distance were correlated ($r=0.76$, $P<0.01$). Peak $VO_2$ and pre-exercise serum TAC were not correlated ($r=0.475$, $P=0.054$). Serum TAC and weekly training distance were not correlated ($r=0.264$). Increments in the plasma concentrations of MDA and CK were not correlated ($r=0.102$).

Following exercise CK, serum βG, plasma MDA, serum TAC, UA and cortisol all showed significant elevations (Table 7.2). As all these biochemical parameters have been corrected for changes in plasma volume, the effects of exercise induced fluid losses have been eliminated. Relative to pre-exercise values UA, and serum TAC increased 17.3±17.6% and 18.8±13.8% respectively, these increases being correlated ($r=0.76$, $P<0.001$).

**Table 7.1 Oxygen uptake, exercise intensity and blood lactate during exercise.**

| | Pre exercise | 5mins @ 50% $VO_2$ peak | 5mins @ 70% $VO_2$ peak | 10mins @70% $VO_2$ peak | 4kms | 8kms | 12kms | 16kms | 20kms | Post exercise |
|---|---|---|---|---|---|---|---|---|---|---|
| $O_2$ uptake ($l.min^{-1}$) | - | 2.25±0.20 | 2.99±0.26 | 3.02±0.28 | 3.32±0.26 | 3.37±0.29 | 3.38±0.28 | 3.43±0.30 | 3.47±0.32 | - |
| $\%VO_2$ peak | - | 51.4±2.3 | 68.1±2.4 | 68.6±2.5 | 75.7±4.3 | 76.8±4.2 | 77.0±4.4 | 78.0±4.0 | 78.0±3.8 | - |
| Lactate ($mmol.l^{-1}$) | 1.17±0.48 | 1.47±0.43 | 1.50±0.52 | 1.35±0.60 | 2.04±0.90 | 2.42±1.10 | 2.67±1.12 | 2.73±1.17 | 3.04±1.40 | 3.84±1.87 |



Note All above data were collected by D.M. Wilkinson and J.L. Fallowfield.

**Table 7.2 Changes in biochemical variables in response to a simulated half marathon run.**

| | Pre exercise | Post exercise |
|---|---|---|
| Creatine kinase ($IU.l^{-1}$) | 98 ± 67 | 133 ± 89**** |
| β-Glucuronidase (kSigma Units.$l^{-1}$) | 15.39 ± 5.34 | 17.05 ± 5.7*** |
| Malondialdehyde ($\mu mol.l^{-1}$) | 1.48 ± 0.39 | 1.65 ± 0.32* |
| Cortisol † ($nmol.l^{-1}$) | 339 ± 95 | 557±157** |
| Antioxidant capacity ($\mu$mol Trolox $Eq.l^{-1}$) | 475 ± 84 | 564 ± 113**** |
| Uric acid ($\mu mol.l^{-1}$) | 268 ± 45 | 312 ± 51*** |

*$P$<0.05, **$P$<0.01, ***$P$<0.001, ****$P$<0.0001 relative to pre-exercise.

† Serum cortisol measurements were performed by D.M. Wilkinson and J.L. Fallowfield.

## Discussion

Increased oxygen utilisation has been shown to produce a similar rise in the production of reactive oxygen species from the mitochondria (Loschen et al. 1973, Boveris & Chance 1973, Pryor 1987). During the half marathon run there was a sustained high rate of oxygen uptake, and fractional utilisation of peak $VO_2$ (Table 1). This suggests that the exercise was intense and produced a considerable increase in ROS. However oxygen uptake probably underestimates the ROS incurred during such intense exercise, due to reduced mitochondrial respiratory control (Davies *et al.* 1982, Sjodin *et al.* 1990) and increased free radical formation from non-mitochondrial sources (Hebbel & Eaton 1989, Symons 1988, Sjodin *et al.* 1990, Sahlin *et al.* 1991, Jenkins 1993). The activity of antioxidant enzymes requiring NADH and/or NADPH as co-factors may also be compromised during intense exercise (Lovlin *et al.* 1987), which would further contribute to ROS during such activity.

Major antioxidants which protect against free radicals in human serum include ascorbate, uric acid, tocopherol, β-carotene, glutathione and albumin (Ames *et al.* 1981, Frei *et al.* 1989, Kretzschmar *et al.* 1991, Whitehead *et al.* 1992, Miller *et al.* 1993, Maxwell *et al.* 1993, Kanter *et al.* 1993). In the trained runners studied, there was a trend suggesting pre-exercise serum TAC was positively correlated with peak $VO_2$, but not weekly training distance. This may indicate that resting serum TAC is elevated in response to long term aerobic training. An increase in UA was observed following exercise, which may have contributed to the rise in serum TAC. Based on the stoichiometric free radical quenching capacity of Trolox and uric acid (Whitehead *et al.* 1992) the rise in UA was estimated to account for only one third of the rise in serum TAC. Previous studies indicate other antioxidants can be elevated during exercise (Gleeson *et al.* 1987, Duthie *et al.* 1990, Viguie *et al.* 1993), which may account for the rise in TAC not attributable to uric acid. As in the present investigation there was a rise in serum cortisol (Table 2), and such changes are correlated with elevations in serum ascorbate (Gleeson *et al.* 1987), it is conceivable ascorbate was elevated during the half marathon.

A rise in UA has been regarded as a beneficial response during physical activity (Ames *et al.* 1981), which may minimise the damage caused by ROS by increasing serum TAC. The increase in circulating uric acid during exercise is not thought to be caused by reduced glomerular filtration (Castenfors 1967), but results from an increase in production (Harkness *et al.* 1983, Sjodin *et al.* 1990). In the present study, lactate induced inhibition of renal uric acid clearance (Emerson 1978) may also have contributed to the accumulation of uric acid in serum.

The increase in serum βG suggests that the half marathon resulted in the release of lysosomal contents. Lysosomal membrane rupture could occur as a consequence of peroxidation (Fong *et al.* 1973), and might be initiated by free radicals produced during exercise. There are a variety of interrelating pathways through which the release of lysosomal contents could cause further increases in free radical production. One mechanism involves the protease catalysed conversion of xanthine dehydrogenase to xanthine oxidase, thereby increasing the production of uric acid and oxygen radicals (Sjodin *et al.* 1990). The net release of hypoxanthine from active muscle suggests this pathway may be a source of free radicals during intense exercise (Sahlin *et al.* 1991).

Before the half marathon CK was similar to previous reports for trained runners (Robertson *et al.* 1991). As CK was slightly higher than observed in sedentary subjects, this may provide evidence for training induced muscle damage prior to exercise (Robertson *et al.* 1991). Elevated CK at rest is consistent with histological observations in the *gastrocnemius* muscle of trained runners, which showed disruption of the sarcolemma and necrotic fibres prior to exercise (Hikida *et al.* 1983). The post-exercise rise in CK provides evidence for increased sarcolemmal permeability, which during running could arise from sarcolemmal membrane rupture (Hikida *et al.* 1983).

Membrane peroxidation is a recognised mechanism of enzyme release in ischaemic reperfusion injury of cardiac muscle (Gauduel *et al.* 1989), and ROS can directly contribute to the disintegration of the cell membrane structure (Wu *et al.* 1996). Significant correlations between CK and MDA following an 80 km race (Kanter *et al.* 1988) may provide evidence that similar mechanisms of enzyme release also occur during running. However in the present study the post-exercise rise in plasma MDA and CK were not correlated, which may indicate the rise

in CK resulted from mechanical trauma rather than lipid peroxidation. In red blood cells, peroxidation products (such as MDA) can decrease membrane fluidity (Pfafferott *et al.* 1982) by forming intra and intermolecular linkages in the cell membrane (Pfafferott *et al.* 1982, Wu *et al.* 1996). Similar cross linking can also occur in the sarcolemmal membrane during exercise (Rajgura *et al.* 1993). Such events might increase the susceptibility of the sarcolemma to rupture during mechanical loading. The absence of elevated CK following exercise which imposes substantial ROS, but does not produce muscle trauma (Ahlborg & Brohult 1967, Berg & Haralambie 1978, Viguie *et al.* 1993), may also provide evidence for a mechanically mediated release of creatine kinase during running.

A large reserve in the capacity to handle free radicals during exercise has been proposed (Viguie *et al.* 1993). In this study the rise in serum TAC may have increased resistance to free radical damage. Despite these responses in serum, the rise in the plasma MDA concentration suggests these adaptations were insufficient to prevent exercise induced lipid peroxidation. This may indicate inadequacies in the antioxidant defence system at the sites of radical formation during prolonged high intensity aerobic exercise.

## Conclusions

In trained subjects performing a simulated half marathon, there was an enhanced ability to quench free radicals in serum. Despite this response there was still an exercise induced increase in the plasma MDA concentration.

# CHAPTER 8

## General discussion

## 8.1 Preface

Interpretation of the experimental findings have already been presented at the end of each research chapter. Therefore the objectives of the general discussion are to 1) propose experimental modifications for the manipulation of strain and force in human muscle 2) consider methodological issues associated with the assessment of lipid peroxidation and antioxidant status in biological fluids 3) evaluate the novel findings in the context of current muscle damage theory, with specific emphasis on possible biochemical mechanisms of exercise induced muscle damage in humans and 4) provide direction for future research on free radical induced tissue damage.

## 8.2 Experimental modifications for the manipulation of strain and force in human muscle

In chapters 3 and 4 experimental evidence was presented that muscle strain and muscle force generation were associated with the initiation of muscle damage. Strain rate may also be a contributing factor to muscle damage (Warren *et al.* 1993), although this has not been evaluated in humans. These authors provided evidence that strain rate was directly related to several muscle damage indices, possibly as a consequence of the visco-elastic properties of muscle. Although several mechanical factors appear to be involved in the initiation of damage, it is unclear from current investigations if this occurs on a continuum, or is the consequence of exceeding particular thresholds. Future work may help to elucidate these phenomena by using a range of muscle forces and strains. Using the techniques described in Chapters 3 and 4, it is unlikely that either strain or force could be manipulated much beyond the ranges used in these studies. The magnitude of KE strain produced is limited by the range of motion of the knee joint. As exercise at 'short' and 'long' muscle lengths (Chapter 3) utilised a range of motion close to full extension and full flexion respectively, it is not possible to produce strains with a much greater magnitude of difference *in vivo*. Human skeletal muscle can be stimulated outside the physiological range of motorneuron firing frequencies reported by Bigland-Ritchie *et al.*

(1983a). Despite this, upper (Edwards 1978, Jones *et al.* 1979, Metzger & Fitts 1987) and lower limits exist for the production of muscle force using PES. Stimulating the human KEs at frequencies above 100Hz does not result in additional force production (unpublished observations). Applying PES to KEs at low frequencies (below 20Hz) may result in a large number of fibres not reaching fused tetanus. This will confound the production of low muscle forces, as a large number of fibres will produce varying forces during the contraction and relaxation phases of pulsatile stimulation. Subjects often perceive low frequency stimulation as painful, therefore subject compliance may also limit the use of PES to produce low muscle forces.

## 8.3 Methodological issues associated with the assessment of lipid peroxidation and antioxidant status in biological fluids

The highly reactive nature of free radicals and free radical products, can make *in vitro* measurements difficult to interpret. Values for the concentration of antioxidants and peroxidation products determined in biological samples are highly assay dependent (Knight *et al.* 1987, Wade & van Rij 1988, Young & Trimble 1991). In part, this may be a consequence of free radical formation during sample collection and preparation. This may result in a consistent overestimation of oxidation products and an underestimation of the *in vivo* antioxidant concentration. Methodological considerations when assessing the MDA concentration in biological fluids and tissues have been discussed previously (Wade & van Rij 1988, Halliwell & Chirico 1993). Assays for MDA measurement which involve heating the sample under acidic conditions (e.g. Knight *et al.* 1988, Conti *et al.* 1991, Young & Trimble 1991) overestimate the concentration of MDA *in vivo* (Wade & van Rij 1988, Halliwell & Chirico 1993). Further MDA is thought to arise from the decomposition of lipid peroxides during heating (Wade & van Rij 1988), therefore MDA assessment via such techniques may reflect the magnitude of peroxidation more accurately than the *in vivo* MDA concentration.

The apparent concentration of MDA in biological fluids may be related to the concentration of free iron, as Wade and van Rij (1988) showed addition of $Fe^{3+}$ to human plasma increased the apparent MDA

concentration. Both heating and acidity might denature plasma proteins involved in the sequestration of metal ions e.g. albumin, transferrin and caeruloplasmin. Thus, a naturally occurring increase in the iron concentration of biological samples, might result in the overestimation of peroxidation *in vivo*. There are several events which occur following damaging exercise which might increase the iron content of blood plasma.

Elevations in serum creatine kinase activity are usually accompanied by the release of myoglobin (Milne *et al.* 1988, Rodenburg et al. 1994) which contains iron. In both studies which reported an elevation in plasma MDA (Chapters 6 & 7) serum creatine kinase activity was also elevated. Thus, although the plasma iron content was not determined directly, in these studies plasma iron may have been elevated at the same time points as CK. Distance running is reported to increase lysis of red blood cells (Eichner 1985, Smith 1995) thereby elevating plasma haemoglobin. As this protein contains iron, the plasma iron content may also have been elevated *via* this mechanism following the simulated half marathon (reported in Chapter 7).

Therefore it could be argued the rise in MDA (reported in Chapters 6 and 7) was an artifact of iron catalysed peroxidation during sample preparation. However this possibility appears unlikely for several reasons. Plasma MDA was not elevated on day 3 after exercise (Chapters 5 and 6) despite the possibility that plasma myoglobin (and therefore iron) was elevated in the sample. This may provide indirect evidence to suggest plasma metal iron chelaters may not release iron during sample preparation. The possibility that serum ascorbate was elevated immediately following the simulated half marathon has previously been discussed (Chapter 7). If free iron was present in the sample ascorbate would be expected to act as a pro-oxidant *via* mechanisms previously outlined in section 1.0.2. However not all subjects who demonstrated a rise in serum TAC and CK (and presumably ascorbate and iron respectively) in chapter 7, showed a rise in plasma MDA. Of the 17 subjects studied, two showed a decline in plasma MDA of 5% or more, following correction for plasma volume changes. It is possible that some of the iron chelating proteins present in the plasma samples were not denatured during sample preparation and therefore did not allow the free iron concentration to rise. Under these conditions the rise in serum TAC during aerobic exercise (Chapter 7)

may inhibit the formation of peroxidation products and thereby reduce the apparent MDA concentration. Such observations are consistent with those of Lovlin *et al.* (1987), who reported moderate intensity cycling exercise (below 70% $VO_2$ max) produced a small decline in plasma MDA.

## 8.4 Evaluation of the novel experimental findings in the context of current muscle damage theory

In Chapters 5 and 7 the TAC of tissue extracts was used as an *in vitro* index of free radical protection provided by chain breaking antioxidants. Many sacrificial molecules (e.g. glutathione and ascorbate) are involved in redox cycling reactions which are enzyme dependent (Packer 1992, Ji 1995). The low concentration of antioxidant enzymes in extracellular fluids may make the method of determining total antioxidant capacity described by Whitehead *et al.* (1992) a valid marker of extracellular antioxidant protection *in vivo*. In tissue, enzymes associated with the redox cycling of important sacrificial antioxidants such as glutathione reductase, are highly concentrated (Harris 1992). Further work is needed to determine if the method used to assess changes in muscle TAC (Chapter 5), are sensitive to physiological changes in antioxidant enzyme activity.

Despite evidence that exercise can initiate oxidation of skeletal muscle proteins (Saxton *et al.* 1994), alone, such free radical mediated damage may not to be sufficient to produce muscle necrosis. To observe this effect mechanical damage to structures associated with the maintenance of cellular homeostasis may also be necessary. The sarcoplasmic reticulum (SR) and sarcolemma (SL) can be considered such structures, as they are responsible for maintenance of low cytosolic and intracellular calcium concentrations respectively.

Damage to these cellular components could be metabolically or mechanically mediated. There is histological evidence to suggest sarcolemmal lesions can occur during exercise in man (Hikida *et al.* 1983) and rodents (Armstrong *et al.* 1983). Such disruption may initiate the processes which ultimately lead to cell necrosis (Wu *et al.* 1996). Although the potential for exercise induced damage to the SR is recognised (Byrd 1992, Jakeman & Maxwell 1993), there is little direct

evidence for this. Damage to either the SR or SL might allow diffusion of ions down their respective concentration gradients thereby elevating cytosolic calcium.

Using *in vitro* exercise protocols contractile activity has been shown to elevate intracellular calcium (Claremont *et al.* 1984). There is also indirect evidence for increased cytosolic calcium in human muscle damaged by exercise. An increase in spontaneous elbow flexion has been reported following eccentric exercise with the arm biceps (Saxton *et al.* 1994, Clarkson *et al.* 1992). This may be indicative of calcium induced contracture in the arm flexors, which is consistent with the reports of hypercontracted sarcomeres following marathon running (Hikida *et al.* (1983). These authors also observed mitochondrial paracrystals immediately following and several days after exercise. Similarly, Friden (1984) reported paracrystals 4 hrs after eccentric cycling exercise in humans. Such structures are thought to be precipitated calcium phosphate (Gillis 1985), possibly as a consequence of mitochondrial calcium overload (Armstrong *et al.* 1991). These observations suggest damaging exercise in humans may produce prolonged elevations in mitochondrial and possibly cytosolic calcium. Although mitochondria play a key role in buffering intracellular calcium (Halestrap *et al.* 1993, Harrington *et al.* 1996), when present in high concentrations this may depress mitochodrial function (Wrogemann & Pena 1976) and increase the formation of reactive oxygen species (Beal 1996).

There is considerable debate whether free radicals initiate or are a manifestation of tissue injury (Jackson *et al.* 1990, Bracci 1992). Increased mitochondrial free radical formation as a consequence of calcium overload may be the first point at which free radicals are involved in necrotic tissue injury. As this event may occur subsequent to cytosolic calcium elevation, free radicals may be considered a manifestation of tissue injury. However the production of reactive oxygen species also has the potential to produce oxidative injury, which may initiate further muscle damage. For example loss of mitochondrial respiratory control has been closely associated with lipid peroxidation (Davies *et al.* 1982) and the release of myocellular enzymes (Gauduel *et al.* 1989). Thus in eccentrically exercised muscle, free radical formation may be increased by metabolic aberrations in damaged organelles such as mitochondria. These events might explain the rise in MDA on days 1 and 2 after damaging eccentric exercise (Chapter 6) and might also

result in the production of immune system chemoattractants (McCord & Roy 1982).

As neutrophils and macrophages produce free radicals, it might be predicted the invasion of such cells would further increase oxidative damage. However the data for Chapter 5 showed cellular infiltration did not elevate indices of free radical injury, or compromise muscle antioxidant status. Furthermore at the time point when myocellular inflammation would be expected to commence in humans, the plasma MDA concentration returned to its' baseline value (Chapters 5 and 6). This suggests that rather than increasing oxidative stress cellular infiltrates may reduce free radical stress in damaged tissue. Although, this proposal is controversial, there is indirect evidence that this might occur in both rodents (Duarte *et al.* 1994) and humans (Meydani *et al.* 1993, Chapter 6).

Duarte *et al.* (1994) examined the effects of a leukocyte inhibitor (colchicine) on cellular infiltration in mouse muscle following stressful treadmill running. These authors also evaluated the muscle content of reduced glutathione, which they proposed could be used as an index of free radical stress. Animals treated with colchicine showed less cellular infiltration and lower reduced glutathione concentrations than control exercise animals. This suggests that in control exercise animals cellular infiltration reduced muscle oxidative stress relative to the colchicine treated animals. Duarte *et al.* (1994) proposed cell detritus contributed to the formation of reactive oxygen species, thus its removal by phagocytes reduced free radical stress in the damaged muscle tissue. Interestingly phagocytosis of mitochondria has been reported following muscle damaging exercise in humans (Hikida *et al.* 1983) and damaged mitochondria could be an important source of reactive oxygen intermediates. If free $Fe^{2+}$ also contributed to free radical formation prior to cellular infiltration, this may have been sequestered by the preventative antioxidant lactoferrin released by neutrophils.

Despite the potential for uncontrolled destruction, the utilisation of free radicals by neutrophils and macrophages appears to be well orchestrated. At the time points when cellular infiltration would be expected to commence in muscle, MDA returned to pre-exercise levels (Chapter 6). The MDA concentration in muscle and plasma also did not rise in response to cellular infiltration (Chapter 5). Serum CK activity also peaked much earlier than the peak in cellular infiltration (Chapter

5). Therefore results of chapters 5 and 6 suggest in young healthy adults the inflammatory response in normal subjects is co-ordinated to prevent peroxidative damage and the continuous degradation of the invaded tissue. The work of Saxton *et al.* (1994) which showed protein carbonyl formation during concentric exercise did not affect muscle damage indices, provides evidence that free radicals do not initiate muscle damage during exercise of short duration. In summary, during discrete bouts of exercise free radicals do not appear to initiate the myocellular injury which results in progressive cellular degradation; but may produce additional tissue injury following exercise, prior to tissue inflammation.

A schematic model outlining possible interactions between events associated with eccentric exercise induced muscle damage is shown in Fig. 8. Conceptually this damage model has some similarities to that proposed by Armstrong *et al.* (1991), although the authors recognised that there were limited data to support a role for free radicals in exercise induced muscle damage. Based on the experimental findings from Chapters 5 and 6, potential roles of free radicals in the damage process are included in the muscle damage model proposed. In relation to the model of Armstrong *et al.* (1991) free radicals appear to be involved in what the authors termed the "calcium overload phase" and "autogenic phase".

The model of muscle injury proposed may provide possible explanations for several commonly observed phenomena, including preferential damage to slow and fast twitch fibres in rodents (Armstrong *et al.* 1983) and humans (Friden *et al.* 1983) respectively. In humans, glycolytic fibres typically have a low oxidative capacity and contain fewer mitochondria than more oxidative fibres (Holloszy & Booth 1976). As mitochondria are important in the buffering of cytosolic calcium (Halestrap *et al.* 1993, Harrington *et al.* 1996), the potential for cytosolic calcium accumulation may be increased if the SR or sarcolemma of glycolytic fibres were damaged. Thus, in glycolytic fibres the lower relative mitochondrial volume may allow the accumulation of cytosolic calcium to concentrations where autogenic processes are initiated. As fast-twitch red fibres from rodents have a greater oxidative capacity than slow-twitch fibres, the reverse being true for humans (Holloszy & Booth 1976), these events may provide an explanation for selective fibre injury following damaging exercise.

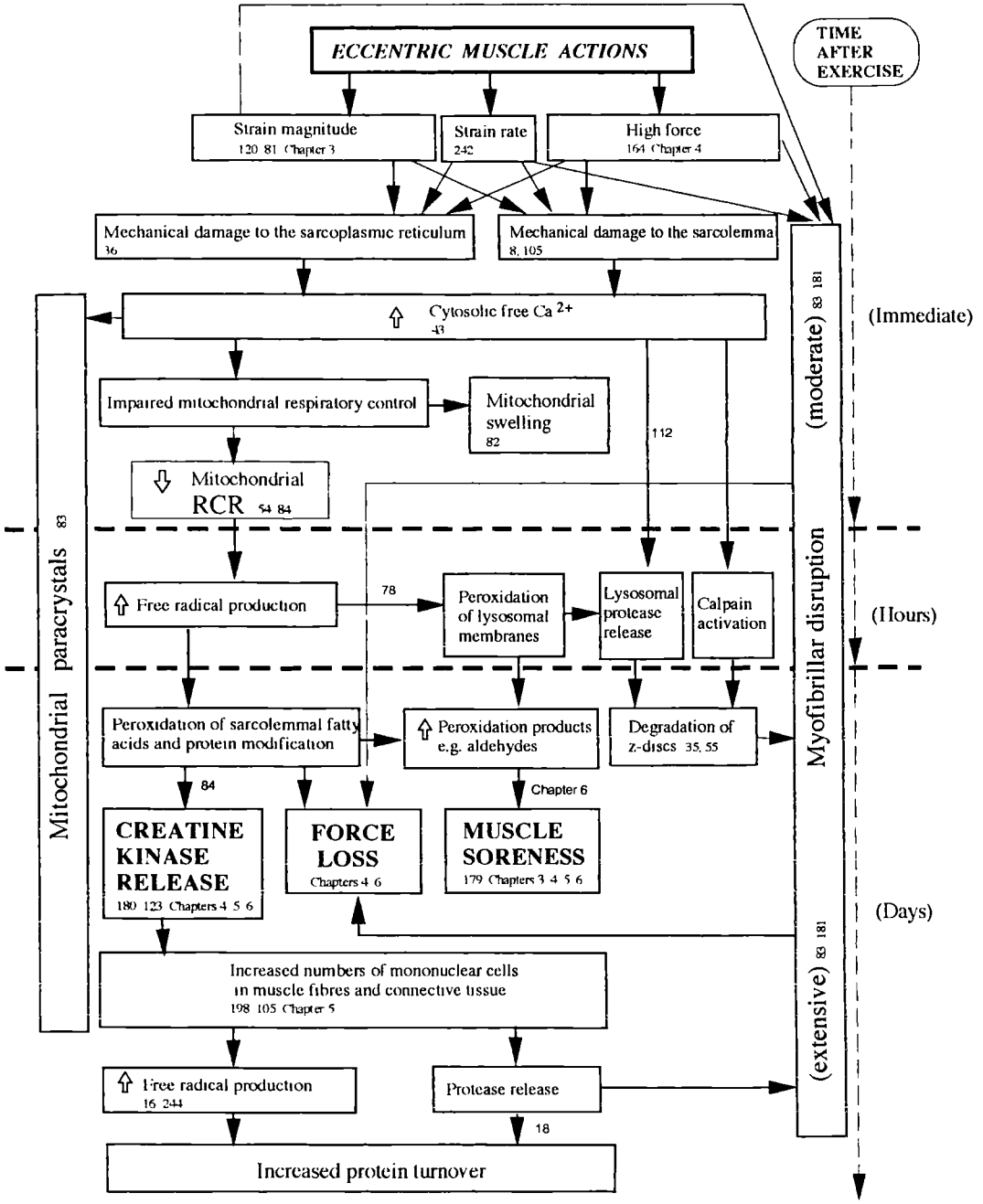


**Fig. 8 Speculative schematic model, indicating possible interactions between events associated with eccentric exercise induced muscle damage.**

Commonly reported manifestations of muscle damage are in capitals. Numbers correspond to supporting references. A number associated with an arrow signifies a recognised pathway. Numbers associated with a box represent the occurence of a specific event.

RCR= respiratory control ratio

Note. Although free radical stress is increased via mitochondrial and inflammatory mechanisms, the latter may not result in free radical damage in humans, as muscle antioxidant status is enhanced.

## 8.5 Possible directions for future research on free radical induced tissue damage

The majority of studies evaluating free radical biochemistry have used indirect methods to assess free radical activity. Although such techniques have been greatly improved, the specificity of some assays is still debated (Halliwell & Chirico 1993). There are still major issues to be resolved regarding the validity of *in vitro* techniques for free radical and antioxidant assessment in complex biological systems.

Many studies could be improved by directly assessing free radical stress and damage indices directly in the tissue. Although this approach has been used in some human studies (Meydani *et al.* 1993, Saxton *et al.* 1994, Chapter 5) there are still limitations to such techniques. Possibly the most important is the inability to distinguish between biochemical changes in damaged and undamaged fibres in crude muscle homogenates. The dissection and identification of individual damaged and undamaged fibres may help to elucidate some of the biochemical changes which occur during the calcium overload phase. Such techniques might help to identify some of the biochemical factors which initiate muscle degeneration. However application of techniques dependent upon cellular integrity may be limited when evaluating changes in degenerating tissue, as a consequence of increased fibre fragility.

Monoclonal antibody techniques have already been developed to assess products of peroxidative damage (Esterbauer *et al.* 1991). Immunohistochemical techniques might allow biochemical changes to be evaluated even in the most damaged muscle fibres. Furthermore, such techniques might allow the location of oxidised structures to be elucidated. Such an approach might help to determine which organelles are producing free radicals and/or being oxidised. The development of immunological techniques to detect oxidised compounds in blood, urine and tissue may also help to overcome problems of assay specificity.

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# Appendices

Appendix 1

## Soreness Questionnaire

Name.....

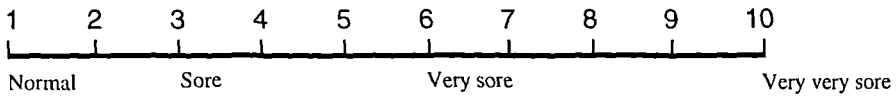
Type of Exercise.....

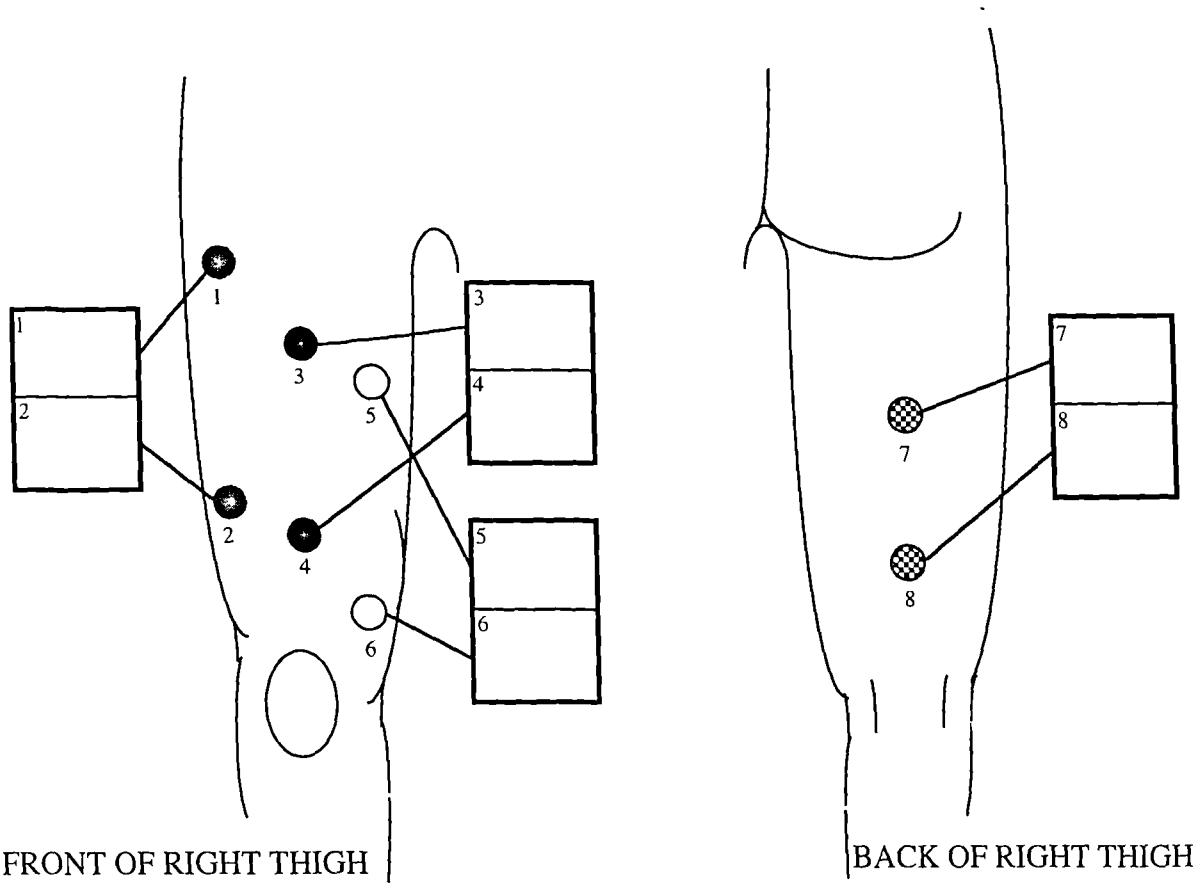
Days Following Exercise.....

Date.....

The purpose of this questionnaire is to evaluate muscle soreness post exercise. Determine the degree of soreness by pressing on the leg muscle at the six sites below

Record the soreness value as a number between 1 and 10 using the soreness scale below as a guide.



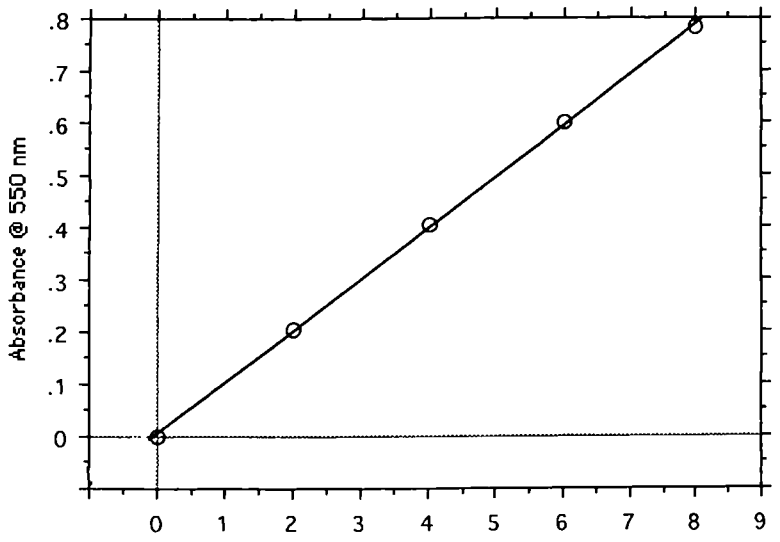




| | |
|---|---|
| Quads mean soreness | - |
| VL mean soreness | - |
| Distal V.L. soreness | - |

RCP 1994

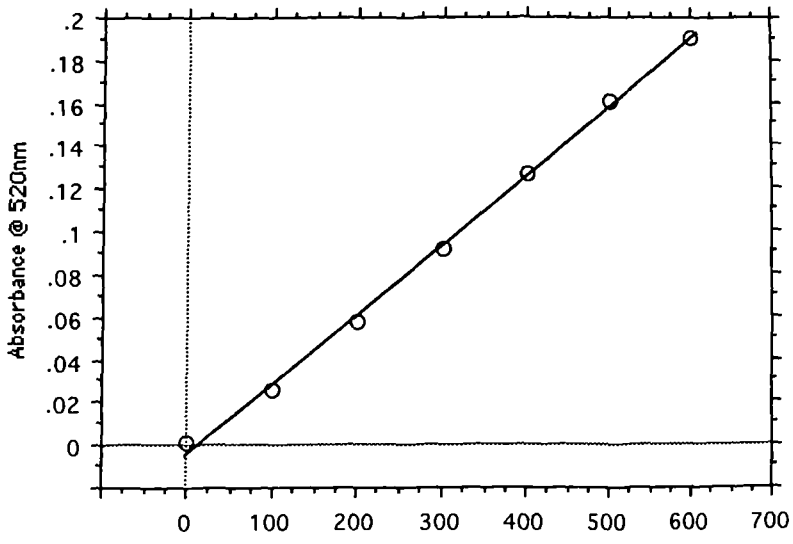
Appendix 2

**Calibration graph demonstrating a linear relationship between phenolpthalein concentration and absorbance.**



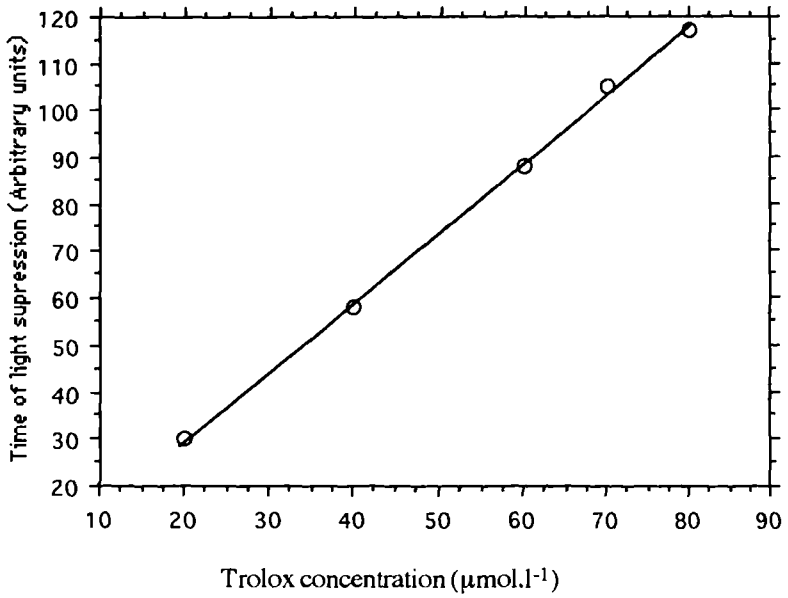
Appendix 3

**Calibration graph demonstrating a linear relationship between absorbance and uric acid concentration.**



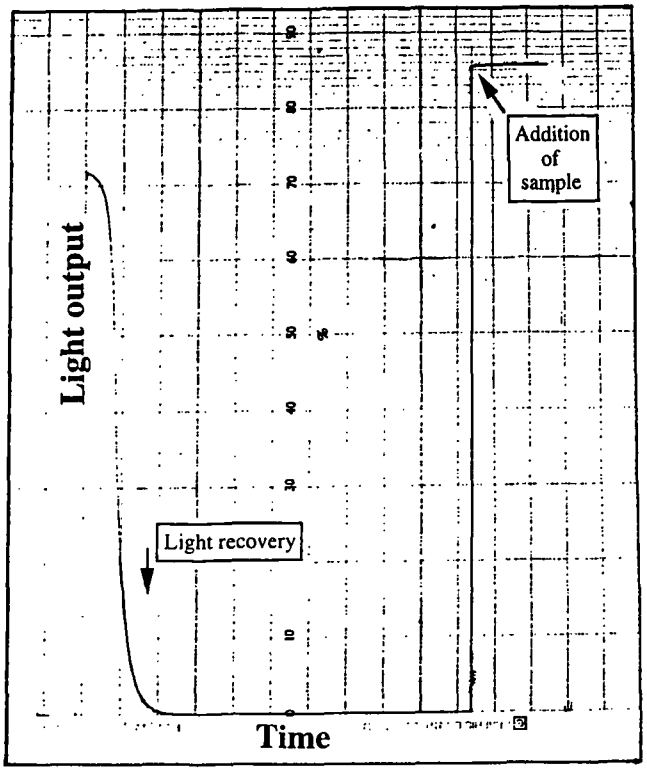
Appendix 4a

**Calibration graph demonstrating a linear relationship between Trolox concentration and time of light suppression.**



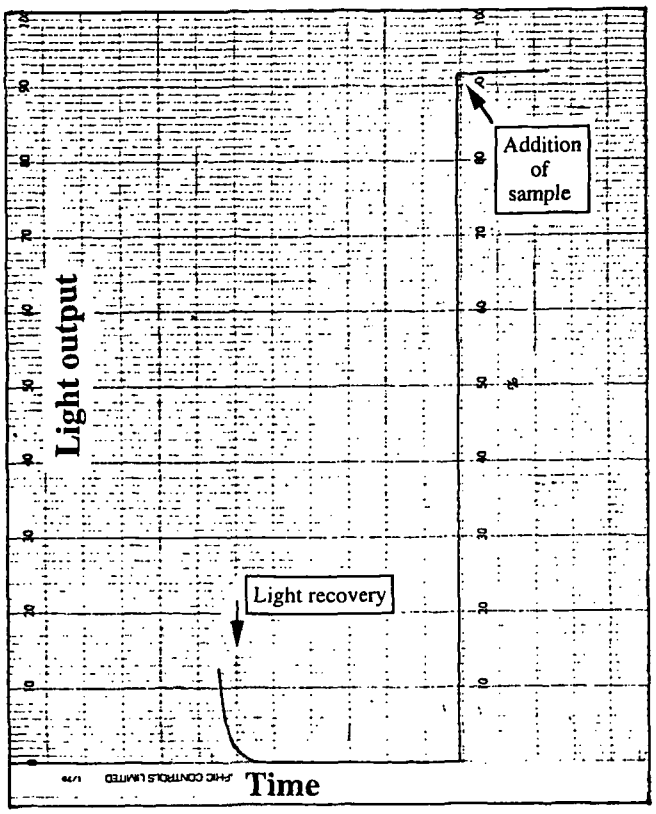
Appendix 4b

**Original record of light recovery following the addition of Trolox to the luminescent solution.**



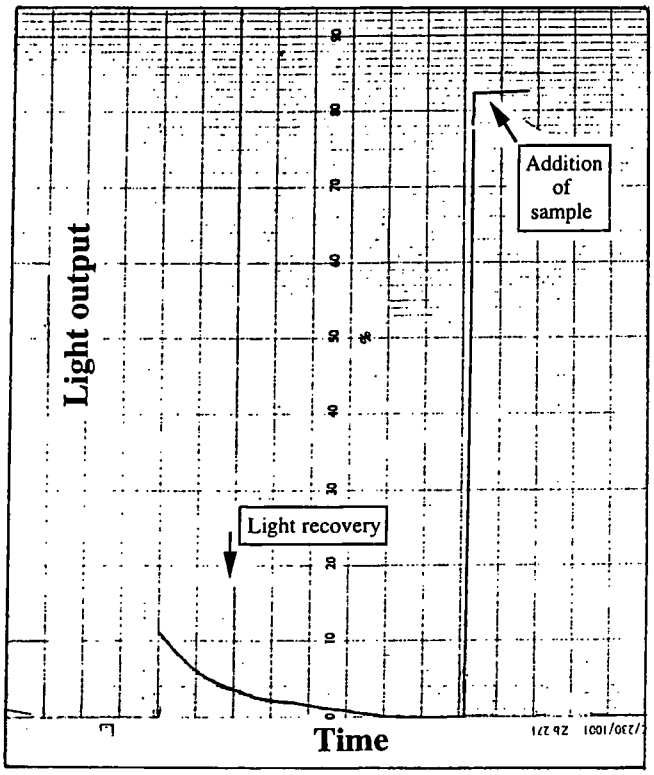
Appendix 4c

**Original record of light recovery following the addition of serum to the luminescent solution.**



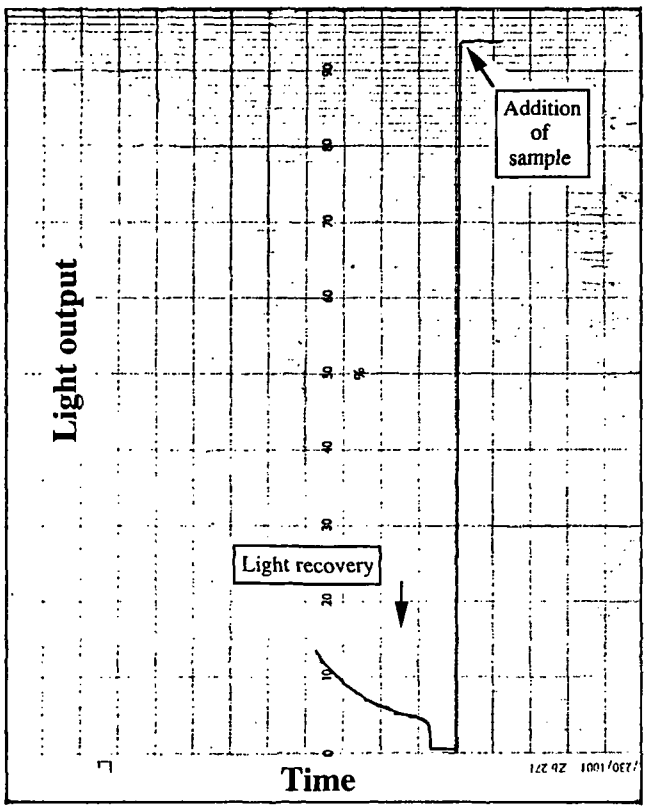
Appendix 4d

**Original record of light recovery following the addition of aqueous muscle extract to the luminescent solution.**



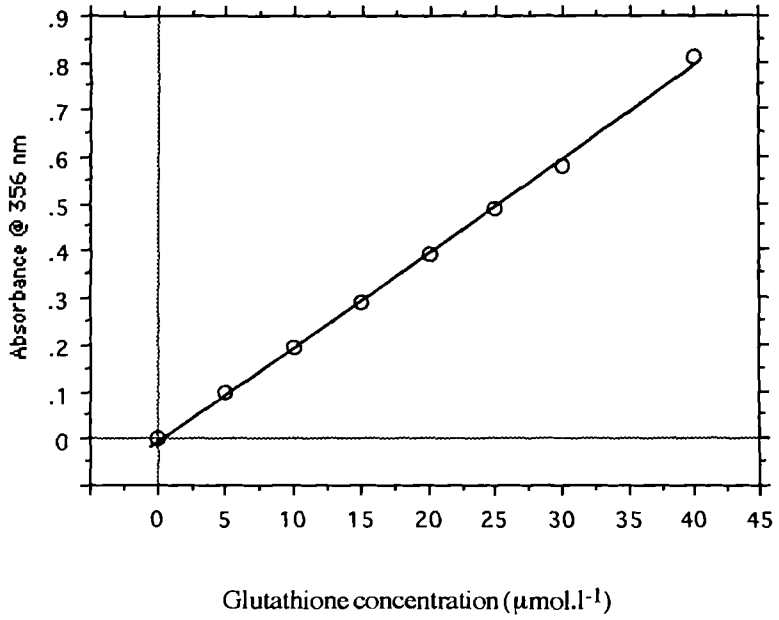
Appendix 4e

**Original record of light recovery following the addition of Trolox to the luminescent solution.**



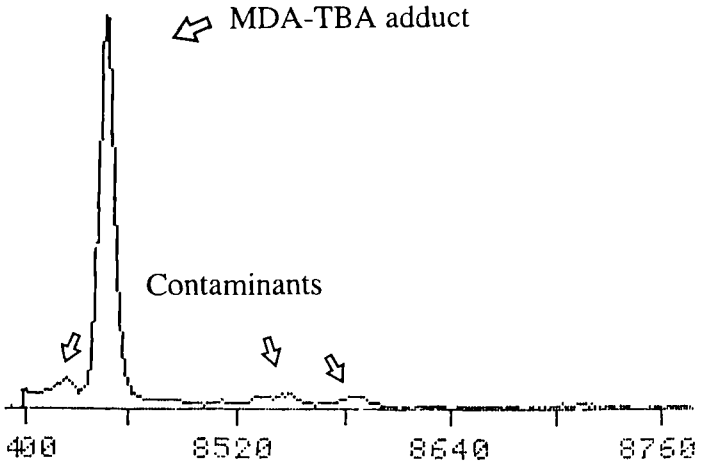
Appendix 5

**Calibration graph demonstrating a linear relationship between sulphydryl concentration and absorbance.**



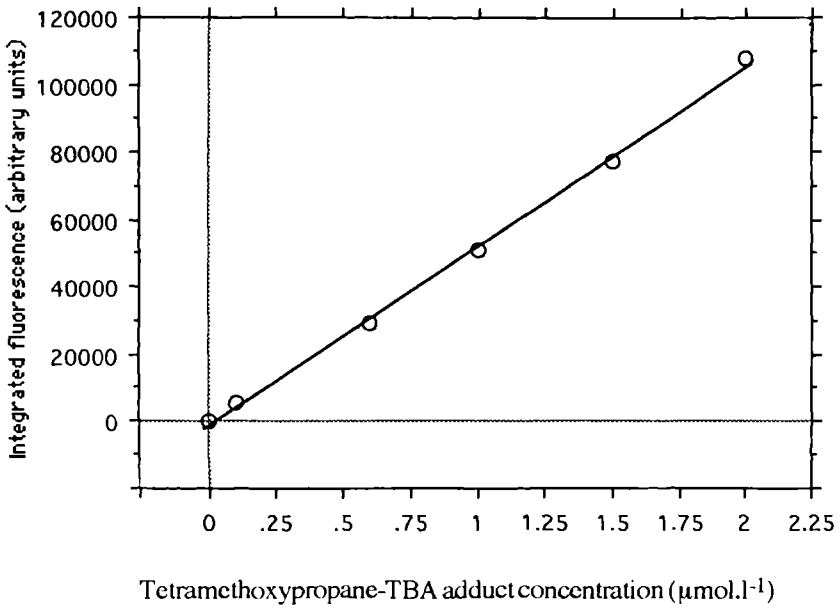
Appendix 6a

**Typical chromatogram showing separation of the MDA-TBA adduct from other fluorescent compounds in plasma.**



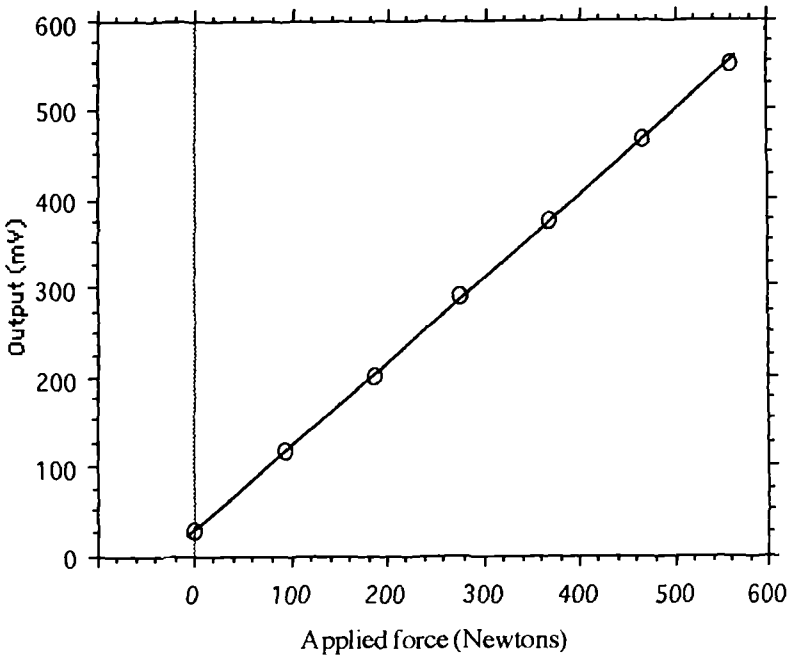

Appendix 6b

**Calibration graph demonstrating a linear relationship between tetramethoxypropane-TBA adduct concentration and integrated fluorescence.**



Appendix 7

**Calibration graph demonstrating a linear relationship between applied force and strain gauge output**



Appendix 8

## Changes in muscle inflammatory indices and antioxidants in response to eccentric exercise.

| Parameter | Baseline | Day 4 | Day 7 |
|---|---|---|---|
| β-Glucurondase<br>(nano gram phenolphthalein formed. hour$^{-1}$ . gram wet weight muscle $^{-1}$) | 89.9<br>(8.3) | 153<br>(20.3) | 312<br>(102) |
| G6PDH<br>(nano mol $NADP^+$ reduced min$^{-1}$ . gram wet weight muscle $^{-1}$) | 63.7<br>(12.9) | 121.7<br>(14.2) | 338.9<br>(120) |
| Total sulphydryls<br>(nano mol. gram wet weight muscle$^{-1}$) | 239<br>(41) | 213<br>(18) | 358<br>(48) |
| Aqueous TAC<br>(nano mol Trolox Eq.gram wet weight muscle$^{-1}$) | 914<br>(117) | 910<br>(76) | 1202<br>(89) |
| Bound extract TAC<br>(μmol Trolox Eq. gram dry weight muscle$^{-1}$) | 24.0<br>(0.06) | 35.5<br>(0.06) | 50.1<br>(0.06) |
| Malondialdehyde<br>(nano mol. gram wet weight muscle$^{-1}$) | 11.3<br>(2.2) | 7.4<br>(1.5) | 13.0<br>(3.5) |